Universidade do Algarve

Faculdade de Ciências e Tecnologia

The role of melatonin in sperm from two aquaculture fish species with reproductive problems: *Solea senegalensis* and *Anguilla anguilla*

Maria Leonor Pires Ferrão

Thesis for Master's Degree in Aquaculture and Fisheries

Specialization in Aquaculture

Dr Elsa Cabrita, CCMAR, University of Algarve

Dr Catarina Oliveira, CCMAR, University of Algarve

2019/2020

Universidade do Algarve

Faculdade de Ciências e Tecnologia

The role of melatonin in sperm from two aquaculture fish species with reproductive problems: *Solea senegalensis* and *Anguilla anguilla*

Maria Leonor Pires Ferrão

Thesis for Master's Degree in Aquaculture and Fisheries

Specialization in Aquaculture

Dr Elsa Cabrita, CCMAR, University of Algarve

Dr Catarina Oliveira, CCMAR, University of Algarve

2019/2020

Declaração de Autoria

"Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída"

_____

Maria Leonor Pires Ferrão

Acknowledgments/ Agradecimentos

Um enorme obrigada à Professora Elsa Cabrita pela sua dedicação admirável ao ensino; pelo seu conhecimento e boa memória; por todas as oportunidades que me ofereceu; por me ter inserido nesta equipa que me acompanhou; pelo tempo que não tem mas que incrivelmente consegue sempre dispor.

À Catarina Oliveira pelo seu otimismo, mesmo nos momentos mais complicados; pela sua calma; pelo seu acompanhamento ao longo deste trabalho; pela sua boa disposição contagiante.

À Víctor Gallego, Marina Morini, Pablo García y Lúz Pérez por vuestra bienquerencia y ayuda preciosa con todo mi trabajo en València; gracias a vosotros por me dejaren acompañaros; por haceren me reír quando me llamaban chinchilla; por haceren me sentir como se estuviera en familia; gracias por vuestro apoyo en los momentos más difíciles.

À minha grande colega e amiga Francisca Félix; por todo o trabalho que realizamos lado a lado; pelas sábias palavras e pelos conselhos; pelos desesperos e alegrias que passamos; pela enorme companhia nos piores e melhores momentos deste ano; pelos ataques de riso; pela tua persistência e preocupação.

Aos meus companheiros e amigos do Aquagroup; à Catarina Anjos; ao Daniel Duarte; ao Diogo Cabeleira; ao Daniel Rodrigues; à Bernardete Lopes; à Elvira Fatsini; a toda a equipa.

Aos meus amigos do coração que são muitos para mencionar aqui, mas que sabem que os tenho comigo.

Aos meus queridos pais; às minhas queridas irmãs; às minhas sobrinhas; à minha avó Lucinda; a toda a minha família.

À ciência e a quem faz dela “tripas coração”.

Um enorme obrigada a todos. Foi um bonito percurso. Que venham muitos mais.

The traineeship performed in the Polytechnic University of Valencia (Universitat Politècnica de València, UPV, València, Spain) was financed by the European Program ERASMUS+.

This work was financed by ASSEMBLE+ JRA2 CRYOMAR project (H2020-INFRAIA-2016-2017, N 730984), to European Regional Development Fund - Interreg Atlantic Area (EBB-EAPA_501/2016 project) and to Mar2020 operational program through project REPROF1 (16.02.01-FMP- 0059). This study was also supported by Portuguese national funds from FCT - Foundation for Science and Technology through project UID/Multi/04326/2020 and from the operational programs CRESC Algarve 2020 and COMPETE 2020.

Abstract

Melatonin can be found in all types of organisms, from multicellular to unicellular. It is widely known for its role in circadian system synchrony, including reproductive cycle control. This molecule is multifunctional, contributing to various cellular mechanisms involving cell homeostasis and oxidative damage protection. Thus, melatonin potential in sperm biology has gain attention as it could improve spermatozoa performance. Although in other vertebrates, melatonin supplementation has exhibited great improvements, in seawater fish species, there are no reports concerning its effects. Therefore, in a first experiment, the effects of melatonin in sperm motility using different concentrations and exposure times were evaluated in two cultured fish species with reproductive dysfunctions, the European eel, and the Senegalese sole. This enabled to better understand the cellular mechanisms by which melatonin can take part in spermatozoa motility. Regarding the European eel, there were no differences in sperm motility among different melatonin concentrations or exposure times, which may be related to MeOH tolerance in this species sperm. In the Senegalese sole, the sperm motility was significantly lower throughout melatonin exposure time suggesting that sperm quality decrease could be allied with DMSO toxicity. However, in 0.1 and 10 mM concentrations, there was a slight increase in spermatozoa motility. Although the motility descriptors were not significantly different, this improvement could be related with melatonin ability to freely cross the spermatozoa membrane cell. A second experiment was conducted to explore melatonin protective effect during sperm cryopreservation in Senegalese sole, following a described protocol for this species as a control versus two melatonin supplementations (0.1 and 10 mM). The post-thawed sperm was evaluated for motility, viability, DNA fragmentation, lipid peroxidation, ROS, and apoptosis detection. The 10 mM melatonin supplement exhibited significantly lower spermatozoa viability combined with higher percentages of late apoptotic cells dead by caspases. Therefore, it suggests that lower melatonin concentrations may provide a better effect in cell protection against apoptotic events triggered by ROS production. Altogether, the obtained results in both experiments emphasize the melatonin species-specificity effect and the necessity of exploring other concentrations with different techniques in fish sperm.

Keywords: melatonin, sperm, Senegalese sole, European eel, cryopreservation.

Resumo

A melatonina pode ser encontrada em diversos organismos, desde multicelulares a unicelulares. É amplamente conhecida pela sua importância no controlo do sistema circadiano, incluindo o controlo do ciclo reprodutivo. Esta molécula é multifuncional, uma vez que contribui para vários mecanismos celulares, como na homeostasia celular e na proteção contra danos derivados do stress oxidativo. Deste modo, o potencial do uso da melatonina na biologia espermática tem ganho interesse, pois demonstrou melhorar a performance dos espermatozoides a diferentes níveis funcionais. Em diferentes espécies de vertebrados, a suplementação do sémen com melatonina traduziu-se numa melhoria na sua mobilidade e viabilidade. No entanto, não existem estudos semelhantes em espécies de peixes marinhos. Como tal, o primeiro objetivo deste trabalho foi precisamente determinar que efeito exerce a melatonina sobre a mobilidade espermática, através da suplementação do sémen com diferentes concentrações de melatonina e durante vários tempos de exposição em duas espécies de peixe com problemas reprodutivos, a enguia Europeia (*A. anguilla*) e o linguado Senegalês (*S. senegalensis*). Neste estudo foram considerados diferentes mecanismos celulares pelos quais a melatonina pode influenciar a mobilidade espermática destas espécies. No caso da enguia Europeia, não houve diferenças significativas na mobilidade entre as diferentes concentrações de melatonina nem entre os tempos de exposição, o que poderá estar relacionado com a tolerância ao metanol, usado nesta espécie para dissolver a melatonina. No linguado Senegalês, a mobilidade foi significativamente menor ao longo do tempo de exposição, o que sugere a perda contínua da qualidade do esperma aliada à toxicidade do DMSO. No entanto, a suplementação de melatonina com concentrações de 0.1 e 10 mM, induziu um ligeiro aumento na mobilidade dos espermatozoides nesta espécie. Embora os parâmetros de mobilidade não tenham sido significativamente diferentes, este aumento pode estar relacionado com a capacidade da melatonina de atravessar livremente a membrana celular dos espermatozoides, atuando em compartimentos subcelulares importantes na performance dos mesmos. Na segunda experiência, o objetivo foi investigar o efeito da melatonina em sémen criopreservado de linguado Senegalês, seguindo um protocolo previamente descrito para esta espécie que serviu como controlo versus duas concentrações de melatonina escolhidas para esta experiência (0.1 e 10 mM).

Os resultados das análises de mobilidade, viabilidade, fragmentação do DNA, peroxidação lipídica, geração de espécies reativas de
oxigénio e deteção de caspases foram utilizados para avaliar o efeito da suplementação da melatonina na solução crioprotetora. A suplementação de melatonina a 10 mM diminui a viabilidade do sémen criopreservado e aumentou a percentagem de células apoptóticas mortas por ação enzimática das caspases, ambas de forma significativa. Portanto, é possível sugerir que menores concentrações de melatonina poderão proporcionar um efeito mais proeminente na proteção celular contra eventos apoptóticos desencadeados pela produção de espécies reativas de oxigénio. Em conjunto, os resultados obtidos nesta tese sugerem que a suplementação do sémen com melatonina pode ter um efeito dependente da concentração e da espécie em causa, de forma semelhante a outros antioxidantes. Para além disso, salienta a necessidade de explorar outras concentrações de suplementação com melatonina, bem como diferentes técnicas de avaliação da qualidade do sémen criopreservado, como por exemplo, o potencial membranar da mitocôndria. Este trabalho representa um ponto de partida para estudos futuros nesta área, que permitirão a otimização da qualidade do sémen criopreservado de peixes, através da suplementação com melatonina.

Palavras-chave: melatonina, sémen, linguado Senegalês, enguia Europeia, criopreservação.

Table of contents

Abbreviations

Amp – Amplitude

ATP – Adenosine Triphosphate

BSA – Bovine Serum Albumin

CASA – Computer Assisted Sperm Analysis

DCF – Dichlorofluorescein

DCF-DA – Dichlorofluorescein Diacetate

DHE – Dihydroethidium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DNAt – Tail DNA

EY – Egg Yolk

FAO – Food and Agriculture Organization

hCG – Human Chorionic Gonadotropin

ICES – International Council for the Exploration of the Sea

IUCN – International Union for Conservation of Nature

LIN – Linearity

MeOH – Methanol

MDA – Malondialdehyde

PBS – Phosphate Buffered Solution

PI – Propidium Iodide

PM – Progressive Motility

s – seconds

SCGE – Single Cell Gel Electrophoresis

SCSA – Sperm Chromatin Structure Analysis

Spz – Spermatozoa

TM – Total Motility

TBAR – Thiobarbituric Acid

TUNEL – Terminal Deoxynucleotidyl Transferase Mediated dUTP-Biotin labelling

mA – Miliamps

min – Minutes

ROS – Reactive Oxygen Species

V – Volts

v/v – Volume/ Volume

VCL – Curvilinear Velocity

VSL – Straight-line Velocity

Index of figures

Index of tables

Introduction

1. Reproduction importance for aquaculture

Fish domestication requires an intense scientific knowledge to overcome several production bottlenecks, with reproduction control representing a considerable effort to achieve aquaculture diversification (FAO, 2018). However, there are still several fish species in aquaculture that exhibit some degree of dysfunction and in these, reproductive control is undoubtedly a production concern. These problems depend on the species and can range from absence of spawning to significant low quantity and quality of produced gametes (Mylonas et al., 2017). Therefore, industrial production of these problematic species often rely on wild captured broodstock, which contributes to the unsustainability of aquaculture (Alavi et al., 2007). Consequently, one of the limiting factors in new aquaculture species is obtaining gametes of proven quality to ensure offspring production and meeting market demand. Thus, the production of high-quality eggs and sperm of novelty species is a prerequisite for the sustainable expansion of aquaculture industry (Lubzens et al., 2017; Mylonas et al., 2017).

In captivity, the control of reproductive function begins with the manipulation of environmental conditions in order to stimulate gamete maturation. However, in many hatchery produced (F1 generation) species, there are important reproductive dysfunctions that hinder a reliable production of eggs and larvae (Mylonas et al., 2010) Although most of these problems are often seen in females, with failure of oocyte maturation, ovulation and spawning, male reproductive performance has also been recognized as a restrictive issue, especially in flatfish species (Morais et al., 2016; Mylonas et al., 2017). In many species with described F1 male reproductive dysfunctions, several methods have been approached to enhance spermatogenesis and, thus sperm production. These include hormonal treatments, environmental manipulation, broodstock nutrition, and sperm management (e.g. sperm cryopreservation). However, the application of these methods constantly requires sperm quality evaluation to better understand the cellular mechanisms by which sperm and overall male reproduction are affected. Thus, the development of tools for male gamete quality evaluation is of great interest in commercial aquaculture both for well-established species as for new ones with industrial potential (Cabrita et al., 2014).

2. Fish sperm physiology

The main components present in fish sperm are spermatozoa and seminal fluid and both are essential for successful reproduction performance. In detail, fish spermatozoa are transcriptionally inactive cells with low cytoplasm content and high polyunsaturated fatty acids (PUFAs) membrane composition, hence possessing an especially vulnerable structure (Cabrita et al., 2019). These features contribute for spermatozoa susceptibility to free radicals or reactive oxygen species (ROS), highly reactive and inherently produced throughout spermatogenesis that can induce serious injury at several cellular levels. Although spermatozoa possess intrinsic antioxidant defences that strictly control reactive oxygen species production, these cells shed a majority of their cytoplasm. Altogether, these innate protective mechanisms become ineffective upon ROS unbalanced generation and, therefore, can compromise sperm motility, viability and consequently successful embryo development (Cabrita et al., 2014).

The seminal plasma plays an important role in fish sperm once it balances these dynamic events by providing spermatozoa protection against ROS during spermatogenesis and further supporting them until their release upon fertilization (Cabrita et al., 2014). The seminal plasma presents enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidants (e.g. glutathione, ascorbic acid, uric acid, taurine). These enzymes are important once they provide intracellular protection of spermatozoa against ROS, whereas extracellularly spermatozoa are protected by both enzymatic and non-enzymatic ROS scavengers (Słowińska et al., 2013). Furthermore, seminal plasma also contains organic (e.g. glycerol, fatty acids, glucose, lactate) and inorganic compounds (e.g. sodium, potassium, calcium) related with energy metabolism and sperm motility processes upon an activation signal (Cosson, 2019). Altogether, the presence of these constituents in the seminal plasma composition is intrinsically related to sperm functionality in terms of spermatozoa performance and fertilization success (Alavi et al., 2019; Dzyuba et al., 2017).

For fish spermatozoa to encounter the egg and fulfil its ultimate goal, which is fertilization, spermatozoa motility needs to be triggered. The differences between the seminal fluid and the spawning surrounding media are the main triggers for sperm motility initiation in most species. Both osmotic or ionic variations lead to flagella movement through depolarization transduction across spermatozoa membrane, perceived

by specific carriers (Dzyuba et al., 2017; Zilli et al., 2017). These interchanges are sustained by the mitochondrial respiration that is responsible for intracellular energetic compound production, such as adenosine triphosphate (ATP) (Cosson, 2019; Dzyuba et al., 2017). It has been reported that spermatozoa motility depends mostly on ATP consumption to sustain flagellar beating, once it becomes available as a substrate for secondary messengers (Dzyuba et al., 2017). The two main secondary messengers described in intracellular transduction pathways in fish sperm motility are cyclic adenosine monophosphate (cAMP) and calcium via calmodulin (Ca^{2+}/CaM). These are responsible by specific proteins activation, respectively protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent kinases (CaMK) (Alavi et al., 2019; Zilli et al., 2017). Although activation mechanisms can vary between species, these generally lead to intracellular ATP concentration decrease at high rate until complete exhaustion at the end of the motility duration (Cosson, 2019).

Nevertheless, fish spermatozoa motility depends on different aspects of the cell, such as mitochondria status, which is essential both for ATP production and ROS generation (Cabrita et al., 2019). In detail, mitochondria are important organelles attending to ATP production through an oxidative phosphorylation mechanism, as mentioned above. This process triggers different metabolic pathways occurring inside mitochondrial microenvironment, such as the decarboxylation of aceto acids, β-oxidation of fatty acids, metabolism of amino acids and pyrimidine synthesis. Simultaneously, the mitochondria are actively involved in other processes, such as the generation of free radicals, apoptosis, and calcium signalling (Figueroa et al., 2016). These redox mechanisms that occur within the mitochondria are the most important source of ROS, which are required for the normal functionality of this organelle. However, oxidative stress situations leading to mitochondrial membrane damage (e.g. cryopreservation) can impair the mitochondrial respiratory efficiency, promoting the release of ROS and triggering an unbreakable cycle in which mitochondria might become both generator and victim of the oxidative damage. Altogether, this oxidative stress situation, provoked by unrestrained ROS liberation, decreases sperm mitochondrial respiration by impairing electron transport and ATP synthesis, which culminate in mitochondrial respiration and ultimate spermatozoa motility decrease (Ferramosca et al., 2013).

The final goal of spermatozoa is the transmission of male genetic information to the embryo and, for this reason DNA integrity and stability should be prioritized in fish

spermatozoa evaluation (Cabrita et al., 2019). During the transformation of spermatogonia to spermatozoa there are various alterations in the chromatic structure which enable cell maturation, the spermatogenesis. These modifications include chromatin structure processing and packaging, DNA and nuclear matrix reorganization, epigenetic pattern remodulation, among many other nuclear adjustments. However, these events have an important contribution to the control of embryo development and in fish spermatozoa, this support is particularly different, once these species display diversified sperm chromatin organization (Herráez et al., 2017). Nevertheless, the organization of these structures is highly susceptible to suffer changes under certain conditions (e.g. cryopreservation) that can alter the DNA packaging and increase stress due to ROS. Thus, these ROS attack can result in DNA fragmentation, which compromise offspring development at several levels (Pérez-Cerezales et al., 2011).

Altogether, these basic but important aspects in fish spermatozoa are commonly employed to evaluated sperm quality and guarantee reliable offspring development for aquaculture expansion. The next subsections will focus on describing the most common evaluation techniques used in fish sperm assessment.

2.1 Spermatozoa motility

Sperm motility is the most commonly used parameter to determine sperm quality and it has been widely applied for sperm evaluation (Kime et al., 2001). Although motility does not represent the complete spermatozoa status, it reveals several other physiology features related to plasma membrane integrity and ATP availability, which elucidate intracellular mechanisms. To measure sperm motility, the computer assisted sperm analysis (CASA) system has been widely employed in fish sperm and provides a quantitative assessment of sperm quality throughout the duration of spermatozoa movement. This computerized system is based on video recording of sperm movement, tracking each individual cell, thus allowing an objective analysis of different motility parameters with high repeatability. Moreover, fish spermatozoa motility parameters provided by CASA system have been widely correlated with fertilization ability (Beirão et al., 2011a). CASA system provides different sperm motility parameters quantification and the most used in fish sperm analysis are related to the average of spermatozoa, as percentage of total (TM) and progressive (PM) motility, parameters related to spermatozoa velocities, as curvilinear velocity (VCL) or straight-line velocity (VSL) and parameters related to individual

spermatozoa trajectories, as linearity (LIN). Specifically, the percentage of motile spermatozoa refers to any cell showing movement while the percentage of progressive motility refers to spermatozoa swimming in a progressive manner. Regarding the velocity descriptors, these include curvilinear velocity (VCL), referred as the actual velocity along sperm trajectory, and straight-line velocity (VSL), referred as the straight-line distance from the start to the end point divided by the tracking time. Moreover, linearity (LIN), which refers to the ratio of net distance moved to total path distance (VSL/VCL) can also be provided and it has been considered a good spermatozoa motility indicator (Gallego and Asturiano, 2018). Altogether, these features make CASA system a valuable tool in fish sperm analysis as it provides a complete motility analysis with great applicability, for instance in cryopreservation protocol evaluation. It enables to compare cryoprotectants, extenders, dilution ratios, cooling ramps and freezing systems efficiency (Kime et al., 2001) and reveals motility pattern differences obtained in fresh and post-thawed sperm and between fish species (Caldeira et al., 2019; Kása et al., 2017).

2.2 Cell integrity and viability

Cell membrane integrity and, thus viability represents one of the easiest methods to evaluate sperm quality. The cell membrane is composed of bilayer of lipids that protects the cell and controls cellular exchanges through transmembrane proteins. Once cellular membrane integrity and permeability are essential for spermatozoa survival, these features are also remarkably vulnerable to cellular damaging procedures (e.g. sperm cryopreservation) (Cabrita et al., 2010a). The most accessible methods of measuring cell plasma membrane viability are through selective dyes permeability or fluorescent probes. The same principle behind these methods is the labelling of viable or non-viable cells, which are quantified through fluorescence microscopy or flow cytometry measurement techniques (Cabrita et al., 2005). Although microscopy requires simpler equipment, flow cytometry allows to accurately evaluate a large number of samples in a shorter time (Cabrita et al., 2008). Moreover, spermatozoa staining using selective dyes (e.g. trypan blue and eosin-nigrosin stains) are being replaced by more advanced methods using the same principle but with fluorescent probes (e.g. propidium iodide (PI) and SYBR-14), once these revealed to be more accurate and, in combination with other fluorescent dyes, can differentiate other cell features besides viability (Cabrita et al., 2014).

2.3 Lipid peroxidation

Lipid peroxidation is a marker based on high content of polyunsaturated fatty acids in fish sperm plasma membrane, which is an ideal target for ROS excessive damage provoked by oxidative stress. This ultimately results in sperm motility impairment and general cell dysfunction, which can be triggered by several practices, such as sperm cryopreservation (Cabrita et al., 2014). Lipid peroxidation is usually assessed by the quantification of malondialdehyde (MDA) which corresponds to the final product of lipid oxidation. The MDA is usually measured by its reactive response with thiobarbituric acid (TBAR) (Cabrita et al., 2014, 2008a), which can also react with other types of compounds besides MDA, making the assay relatively nonspecific. Other commercial kits (e.g. BIOXYTECH MDA-586) have been developed to minimize interferences from lipid peroxidation products and successfully measured MDA production in fish sperm (Martínez-Páramo et al., 2012a). Similarly, fluorescence dyes (e.g. BODIPY®) have been used in fish spermatozoa, once these are incorporated into plasma membrane and emit fluorescence in response to lipid peroxidation measured by flow cytometry (Hagedorn et al., 2012).

2.4 Reactive oxygen species (ROS)

It is known that ROS have a critical role in spermatozoa performance and the most described oxygen species are hydroxyl radical (-OH), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). Although controlled by cellular inherent antioxidant mechanisms that minimize injuring potential, reactive oxygen species reveal high reactivity capacity, which in turn promotes cell injuries at various levels (Cabrita et al., 2014). The ROS generation can be detected by fluorescence measurement techniques based on reactive procedures inside spermatozoa, which emit fluorescence upon oxidation by oxygen species. The application of these fluorescent probes can be advantageous for sperm analysis once the combination with viability dyes (e.g. PI, 7-aminoactinomycin D (7-AAD)) enable to eliminate non-viable population from analysed sperm sample (Martínez-Pastor et al., 2010).

There are few fluorescent probes described in fish spermatozoa for accurate ROS detection. For instance, dihydroethidium (DHE) has been used to quantify intracellular superoxide anion production and it exhibits blue fluorescence in spermatozoa cytoplasm,

but once oxidized, this probe intercalates with DNA content and stains the nucleus with red fluorescent (Hagedorn et al., 2012). Another fluorescent probe is dichlorofluorescein diacetate (DCFH-DA), which penetrates the plasma membrane and remains retained until cleavage by esterases that produces dichlorofluorescein (DCF), emitting green fluorescence upon hydrogen peroxide oxidization. This fluorescent probe has been applied to compare ROS levels in Senegalese sole sperm (Valcarce and Robles, 2016).

2.5 DNA integrity

Attending sperm DNA vulnerability to suffer damage promoted by intrinsic (e.g. apoptosis) or extrinsic (e.g. cryostorage) and its ultimate goal for genetic information transmission to the next generation, it is of greatest importance to ensure its integrity in order to guarantee progeny survival (Herráez et al., 2017).

There are different methods to evaluate DNA integrity, based on chromatin damage through DNA fragments detection, including TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labelling), sperm chromatic structure assay (SCSA) and Comet assay or single cell gel electrophoresis (SCGE) (Cabrita et al., 2010). Briefly, the TUNEL assay relies on fluorescence labelled nucleotide addition to the 3'OH end of the DNA strand and its emission is proportional to free 3´ends presence and consequent DNA fragmentation (Cabrita et al., 2011). SCSA is based on chromatin integrity using acridine orange fluorescence, which emits green and red fluorescence when associated, respectively, to intact DNA strands or denatured DNA. The most commonly employed in fish spermatozoa is the comet assay which analyses chromatin fragmentation based on electrophoretic migration patterns of DNA fragments derived from spermatozoa lysis. After staining with fluorescent DNA-specific stain, DNA can be observed in fluorescence microscopy with disperse DNA fragmented cells forming the comet tail structure, previous to non-fragmented DNA, which completes the comet head. Although comet tail length can be measured manually, there is specific image analysis software for the determination of several parameters (e.g. tail moment, DNA percentage in comet tail) (Cabrita et al., 2014).

2.6 Apoptosis

Cell apoptosis, a programmed cell death mechanism, is a common process during spermatogenesis. It involves a series of biochemical events (e.g. caspase pathway activation and phosphatidylserine exteriorization) mediated by apoptosis-specific enzymes (e.g. initiator caspases-2, 8, 9 and effector caspases-3, 6 and 7) that trigger cellular morphological alterations (e.g. plasma membrane and DNA fragmentation) (Aitken and Koppers, 2011). Thus, the apoptotic cascade activation leads to the expression of these apoptotic markers and eventually proceeds to cellular termination (Said et al., 2010). This apoptosis selective process has a crucial role especially in sperm quality, once the presence of apoptotic cells has been correlated with sperm maturation and motility impairment (Aitken and Koppers, 2011; Said et al., 2010). Therefore, the evaluation of apoptotic events and respective stages in spermatozoa can have great potential in fish sperm quality assessment.

The presence of apoptotic cell population in fish sperm has been documented using fluorescent dyes such as annexin-V (Beirão et al., 2008) or YO-PRO-1 (Beirão et al., 2010). These dyes identify cells with compromised plasma membrane and act as early apoptosis markers. However, more recent studies performed in fish sperm demonstrated that caspase detection can be more specific than other fluorescent dyes (Riesco et al., 2017; Valcarce et al., 2016). In these studies, the detection of active caspases in thawed samples was performed with CaspGLOW™ (Fluorescence Active Caspase Staining) and Muse™ Caspase-3/7 commercial kits. These assays perform caspase detection in different manners. The CaspGLOW™ kit contains a caspase specific inhibitor (i.e. Z-VAD-FMK) directly conjugated with fluorescein isothiocyanate (Valcarce and Robles, 2016). The Muse™ Caspase-3/7 kit contains a DNA binding dye linked to a DEVD peptide substrate that is released upon effector caspases (i.e. caspase-3 and caspase-7) cleavage activity (Riesco et al., 2017). Nevertheless, these methods require contrast staining with viability dyes, such as PI or 7-AAD for membrane compromised cell detection.

3. Fish sperm cryopreservation

Sperm cryopreservation is defined as a long-term technique that preserves spermatozoa cells through temperature decrease and, in some cases, until ultra-low temperatures

(Diogo et al., 2018; Tiersch et al., 2007). This technique is of great interest, not only for fish farming but also for conservation and genetic improvement of biological resources. In fact, the benefits of sperm cryopreservation include synchronization of gamete availability of both sexes, simplification of broodstock management, transport of gametes between fish farms and germplasm storage for genetic selection programs or conservation of species (Cabrita et al., 2010a). Regardless of these advantages, this technique can be challenging, once freezing and thawing procedures induce several cellular damage, which compromise overall sperm quality. Most of these cryoinjuries are related to cold shock, osmotic stress events during cooling, or even ice crystal formation (Cabrita et al., 2008)

In order to establish sperm cryopreservation protocols, many cryoprotectant and extender combinations have been established for several species. These components are crucial to ensure optimal sperm conditions during freezing and thawing processes and, thus sperm cryopreservation success. Briefly, the extenders are essential for sperm dilution to facilitate sperm handling, given several fish species produce high viscosity sperm and often in very low quantity (Muchlisin, 2005). These extenders usually simulate seminal plasma composition and can incorporate plasma membrane stabilizing compounds (e.g. bovine serum albumin (BSA), uric acid) that improve sperm resistance and prevent osmotic stress during cryopreservation (Cabrita et al., 2008a). Regarding cryoprotectants, these are essential for spermatozoa protection and are classified depending on their permeability capability. There are permeable agents, able to enter the cell and replace water content during dehydration process (e.g. dimethyl sulfoxide (DMSO), methanol (MeOH), ethylene glycol) and non-permeable cryoprotectants (e.g. bovine serum albumin (BSA), egg yolk (EY), sucrose) with higher molecular weight, unable to enter spermatozoa but with external protection capacity. It is important to state that cryoprotectants can cause toxicity depending on concentration and equilibrium time before freezing and, therefore an optimal balance becomes crucial for cryopreservation success (Cabrita et al., 2008b). Although to a lesser extent, the supplementation with antioxidant compounds such as aminoacids (e.g. hypotaurine and taurine) and vitamins (e.g. ascorbic acid and α-tocoferol) has also been explored in order to reduce oxidative damage in thawed fish sperm and surprisingly revealed a species-specific protection effect (Cabrita et al., 2011; Martínez-Páramo et al., 2013, 2012a). Besides these beneficial outcomes, there are few studies conducted in fish species and these are limited concerning antioxidant compound diversity.

Generally, sperm cryopreservation has been well developed for freshwater species, such as salmonids (Cabrita et al., 1998; Martínez-Páramo et al., 2009), sturgeons (Horváth et al., 2008) and carps (Horváth and Urba, 2000). However, in the last years several sperm cryopreservation protocols have also been established for many marine species, including the Senegalese sole (Riesco et al., 2017) and the European eel (Herranz-Jusdado et al., 2019b).

4. Species of study

The next sections will focus on describing two fish species of commercial interest and which have male reproduction problems. In both cases, sperm quality evaluation tools described previously enabled a better understanding of their male reproduction performance and, consequently, sperm cryopreservation protocol improvement.

4.1 Senegalese sole

Senegalese sole (*Solea senegalensis*) is a valuable commercial species in Southern Europe, particularly in Spain and Portugal, with great aquaculture potential (Morais et al., 2016). This species is widely distributed in the Mediterranean and Southern Atlantic, being well adapted to moderate climate and industry production initiated in extensive earthen ponds (Dinis, 1992; Dinis et al., 1999). Senegalese sole aquaculture was linked to the salt marshes in the south of Portugal and Spain as an added value product in polyculture with semi-intensive sea bream and sea bass cultivation (Ferreira et al., 2010; Morais et al., 2016). Although salt-marsh culture systems are still used, Senegalese sole aquaculture is trending for more intensive on-shore tank systems, using either shallow raceways or conventional tanks. It is highly appreciated for the organoleptic qualities which, consequently, command considerable market prices (Muñoz-Cueto et al., 2019).

Even though Senegalese sole has been considered an emergent species in aquaculture diversification, progress towards sustainable production has not grown as expected, mostly due to first generation (F1) reproductive dysfunction under captivity and consequent reliance on wild-caught broodstock (Canavate et al., 2009, Muñoz-Cueto et al., 2019b). Most of the problems encountered in the reproduction of this species have been attributed to poor egg quality and low fertilization rates (Cabrita et al., 2006). However, Senegalese sole culture is worsened by the absence of mating behaviour in male F1 breeders alongside low quantity and poor quality sperm production (Cabrita et

al., 2019; Morais et al., 2016). The impairment of quality in sperm produced by F1 males when compared to wild breeders has been verified (Beirão et al., 2011b, 2008; Cabrita et al., 2019). Also, several studies have demonstrated a cellular dysfunction in F1 spermatozoa, once more apoptotic cells have been found and fewer quality sperm transcripts were expressed in comparison with wild-captured males (Guerra et al., 2013; Valcarce et al., 2016).

The absence of natural reproduction of F1 breeders in captivity make artificial fertilization an alternative to sustainably produce Senegalese sole. However, as described above, one of the main problems in artificial fertilization of this species has been the variable quality and quantity of collected sperm. Therefore, sperm cryopreservation of selected high quality samples can be a great tool to overcome this issue (Cabrita et al., 2019; Morais et al., 2016). The most recent protocol developed for wild Senegalese sole in our group by Riesco et al. (2017) yielded the best storing conditions with dimethyl sulfoxide (DMSO) in the cryopreservation media. In this work, both conventional and molecular approaches enabled us to further understand Senegalese sole sperm cryopreservation protocol efficiency. However, more studies should be performed to ensure sperm cryopreservation reliability for artificial fertilization application and, most importantly to adjust cryopreservation procedure to the F1 sperm quality conditions (Riesco et al., 2017).

4.2 European eel

The European eel (*Anguilla anguilla*) is a marine fish distributed across the majority of coastal countries in Europe and along the Mediterranean coasts of Africa and Asia (Tesch, 2003; Tsukamoto et al., 2011). This species displays a peculiar catadromous life strategy, once they spend most of their life in continental waters and migrate thousands of kilometres towards the Sargasso Sea to spawn. After spawning, the breeders die and the larvae, also known as leptocephalus, return to continental waters, where they metamorphose into glass eels (Tesch, 2003). These eels remain in freshwater until they achieve the silvering phase, undergoing a process related to the beginning of puberty and sexual maturation (Aroua et al., 2005). Finally, pubertal silver ells roam again to the Sargasso sea for reproductive migration and the life cycle begins once more (Tesch, 2003).

The populations of European eel have suffered a dramatic decline in the past years due to overfishing, together with other factors as pollution, river dam buildings or pathologies (Stone, 2003). In fact, the ICES (International Council for the Exploration of the Sea) announced that the status of this species remains critical as wild stocks decreased by 99% in the last thirty years, leading to the listing of the species as "Critically Endangered" on the Red List of Threatened Species, by the International Union of Conservation of Nature (IUCN) (Freyhof and Kottelat, 2010). Therefore, to restore wild populations and reduce pressure from eel capture, there is an urgent need to improve management of existing stocks and establish their reproduction under captive conditions (ICES, 2012).

European eel aquaculture is based on the grow-out of juvenile specimens (such as glass eels) captured from the wild, which are stocked in recirculation systems until commercial size. Therefore, the availability of juveniles and adult eels are undeniably limiting factors for eel aquaculture establishment. There were many attempts to reproduce this species in captivity but it has several conditioning factors that make eel production difficult (Asturiano et al., 2004). For instance, European eel reacts slowly to hormonal stimulation used to obtain gametes for artificial fertilization. It can take over 2-5 months until full sexual maturation is achieved and such treatments are very expensive (Herranz-Jusdado et al., 2019c). As of yet, female eels have not responded to such treatments, whereas weekly injections of human chorionic gonadotropin (hCG) for 5-6 weeks proved to mature males, providing high volumes of sperm (Herranz-Jusdado et al., 2018).

In order to store good quality sperm samples and prevent maturation impairment between sexes, several cryopreservation protocols have been developed for European eel (Asturiano et al., 2004a; Peñaranda et al., 2010). The most recent protocol developed by colleagues yielded the best results by combining methanol (MeOH) in the sperm freezing media. Nevertheless, the exploitation of additives may improve spermatozoa protection and consequently overall thawed sperm quality (Herranz-Jusdado et al., 2019b).

5. Melatonin: current and perspective functions in fish reproduction

Fish, like other vertebrates, have evolved in a cyclic environment which is constantly changing. To keep up with these continuous variations, they have developed circadian oscillators to anticipate forthcoming events. These biological clocks are broadly located in fish organs, but the pineal organ is indeed the key component of the circadian system.

The pineal organ is photosensitive once it possesses photoreceptive pinealocytes that code light information into the rhythmic production of chemical messengers, including melatonin (Falcón et al., 2010). Bloodstream plasma melatonin levels rise during the night, establishing a parallel rhythmic pattern with the pineal melatonin levels (Francis et al., 2004). Such melatonin rhythmicity provides crucial information about annual, monthly and daily periodicities and influences biological rhythms (Bromage et al., 2001; Oliveira et al., 2010). For instance, melatonin is thought to act on the hypothalamic-pituitary-gonad axis, controlling reproduction timing through the production of sex steroids and growth factors in the gonads (Oliveira and Sánchez-Vázquez, 2010). Most of the studies regarding this correlation in fish have focused almost exclusively on melatonin production by photosensitive organs, such as the pineal organ (Oliveira et al., 2011, 2009).

Melatonin is an ubiquitous molecule and it can be found in all evolutionary life forms, from multicellular to unicellular organisms (Zhao et al., 2019). Although melatonin is widely known for its importance in many previously described physiological functions, this molecule is considered pleiotropic given its remarkable multifunctionality, which contributes to a variety of cellular mechanisms (Gurer-Orhab and Suzen, 2015). There is evidence that melatonin can target directly cell metabolism through various molecular pathways, which ultimately lead to cell progressive adaptation (Liu et al., 2019). In fact, melatonin activity through membrane or intracellular receptors and as a powerful antioxidant has been widely described, especially in highly metabolic cells, such as neural, epithelial and gastrointestinal cells (Acuña et al., 2014; Li and Zhou, 2015). Melatonin in these cells is important mostly for cell homeostasis regulation and oxidative damage protection, which could lead to cellular degradation (Reiter et al., 2017). Thus, the effect of melatonin in spermatozoa biology has drawn increasing attention, as spermatozoa are structurally susceptible to oxidative damage through lipid peroxidation and DNA oxidation (Aitken et al., 2012; Cebrián-Pérez et al., 2014). In other vertebrates, melatonin supplement promoted sperm functionality by increasing motility and, overall sperm performance (Fujinoki, 2008; Gonzalez-Arto et al., 2016). Also, specific melatonin receptors have been found in spermatozoa of seasonal and non-seasonal breeder species (Gonzalez-Arto et al., 2016). Besides these findings, further studies have demonstrated the beneficial effects of melatonin on sperm cells in humans (Ortiz et al., 2011) and boar (Jang et al., 2010).

In this perspective, it has been demonstrated that melatonin should be involved in the protection of different cells against damage-induced apoptosis by acting as a free radical scavenger (Reiter et al., 2017). Melatonin stimulate antioxidant enzymatic activity involved in radical substances metabolism while preserving cells membrane fluidity. This achieves an extreme importance in sperm cryopreservation, once it is well known that sperm cryopreservation procedures cause irreversible damage at various cellular levels. This damage is mostly caused oxidative cellular stress and leads to reactive oxygen species (ROS) formation, which triggers a cascade of spermatozoa degradation events (e.g. enzymatic caspases activation for apoptosis), thus comprising sperm motility (Aitken et al., 2012; Ashrafi et al., 2013). In other vertebrates, melatonin supplementation in post-thawed sperm resulted in general decrease of oxidative cell damage, intracellular membrane lipid peroxidation, apoptosis markers and DNA fragmentation (reviewed by Len et al., 2019). These protective effects provided by melatonin could be important especially for fish post-thawed spermatozoa, as these possess a high PUFAs membrane content, which is especially a good target for oxidative stress damage (Cabrita et al., 2014). In fish, there are only a few studies regarding the effects of exogenous melatonin in spermatozoa with an improvement in sperm motility (Gao et al., 2019; Lombardo et al., 2014), while no studies focused on the effects of melatonin against cryodamage in fish sperm have been performed. Melatonin supplementation in the freezing media could bring important improvements to fish sperm cryopreservation protocols, considering spermatozoa vulnerability to oxidative stress during such process.

Objectives

The main objectives of this work were to assess the effects of melatonin supplementation in sperm from two fish species, *Solea senegalensis* and *Anguilla anguilla*, and also the effects of melatonin supplementation in cryopreserved sperm of *Solea senegalensis*. Therefore, in order to achieve these objectives was necessary:

- To assess the effects of melatonin concentration and exposure time in terms of sperm motility of F1 *Solea senegalensis* and *Anguilla anguilla* males.

- To elucidate melatonin potential role in fish sperm mechanisms.

- To optimize sperm cryopreservation protocol for F1 *S. senegalensis* established by Riesco et al. (2017) by exploring melatonin antioxidant proprieties.

- To assess different sperm quality parameters after cryopreservation in F1 *S. senegalensis*.

Material and methods

1. Broodstock conditioning

1.1 Senegalese sole

First generation (F1) male broodstocks were established at Ramalhete Experimental Station (CCMAR, University of Algarve, Faro, Portugal), distributed among six outdoor tanks, within a semi-closed system. Stock density in each tank was around 4kg/m^2 (females 1.48 ± 0.23 kg, males 1.02 ± 0.16 kg) and sex ratio 1:2 (females:males). Each fish was tagged with an individual Passive Integrated Transponder (PIT) tag (Trovan). Tanks were supplied with continuous aeration and water exchange was 0.5 m^3/h. Temperature and photoperiod varied according to natural conditions, although threshold temperatures were maintained when required throughout the year. Light intensity was reduced with a shad net placed over the tanks. Broodstock were fed on artificial pellets (SPAROS Lda.) at a daily ration of 1-3% biomass.

1.2 European eel

Eel male broodstock brought from a local fish farm were established at the Aquaculture Laboratory from the Universitat Politècnica de València (UPV, València, Spain) and acclimatized to seawater for 10 days. Eels were kept in 150-L tanks supplied with aeration and equipped with a recirculation system. Each tank had a stock density around 3kg/m^3 (males 1.56 ± 0.14 kilograms). Hormonal treatment for sexual maturation was provided to each male through weekly intraperitoneal injections (hCG, Ovitrelle, Merck; 1.5IU/g fish) over 10 weeks. As eels cease feeding throughout sexual maturation, no fed was supplied during sampling period in order to mimic natural conditions.

2. Sperm collection and evaluation

2.1 Senegalese sole

Sperm samples were collected during spawning season every two weeks from anesthetized individuals in seawater with 2-phenoxyethanol (300 ppm). For experiment

1, sperm samples were collected from the beginning of May to the end of June and for experiment 2, sperm samples were collected from the beginning of May to the end of July. The sperm was obtained by gently pressing the testes on the fish blind side and the urogenital pore was previously cleaned with PBS to prevent contamination with urine, feces, mucus or seawater. Samples were placed in eppendorf tubes and kept on ice in a styrofoam support until further analysis. Contaminated samples with urine, feces or seawater were immediately discarded. Sperm samples were activated by mixing 1 µL of sperm in 10 µL of artificial seawater (pH 8.2). The activation was performed in a Makler chamber using a phase contrast microscope (Nikon 200) with a 10x negative contrast objective connected to a digital camera (ISAS 782M, Proiser R+D, S.L.) set for 50 frames per second (fps). Sperm motility was analysed after activation using computer-assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser R+D, S.L.). For experiment 1, sperm motility was characterized at 15 s post-activation and for experiment 2 at 15, 30, 45 and 60 s post-activation according to total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and linearity (LIN, %). Only samples with total motility above 40% were selected to be used in the experiments.

2.2 European eel

Sperm samples were weekly collected by anesthetizing the individuals in seawater with benzocaine (60 ppm) 24 hours after the hormonal injections. Sperm samples were collected from beginning of February to the end of March. For sperm collection, the genital area was cleaned with freshwater and dried to avoid urine, feces or seawater contamination before applying gentle abdominal pressure. Sperm samples were collected using a vacuum aquarium air pump with plastic falcon attached and were maintained at 4ºC until further analysis. Sperm motility from each collected sample was immediately measured using a dilution of 1:25 (v/v) in P1 medium (125 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 30 mM KCl, pH 8.5) proposed by Peñarada et al. (2010). Sperm samples were activated by mixing 0.5 µL of diluted sperm in 4.5 µL of artificial seawater with 2% (w/v) bovine serum albumin (BSA) (pH 8.2). The activation was performed in a ISAS Spermtrack 10 counting chamber (similar to Makler chamber) using a phase contrast microscope (Nikon Eclipse 80i) with a 10x negative contrast objective connected to a digital camera (ISAS 782M, Proiser R+D, S.L.) set for 60 frames per second (fps). Sperm motility was analysed after activation using computer-assisted sperm

analysis (CASA) and ISAS v1 software (Proiser R+D, S.L., Spain). The same motility descriptors were recorded similarly to *S. senegalensis* sperm. For experiment 1, sperm motility was characterized at 15 s post-activation. Only samples with total motility over 60% were selected to be used in the experiments.

3. Experimental design

3.1 Experiment 1: effect of melatonin concentration and exposure time in sperm motility from F1 *Solea senegalensis* and *Anguilla anguilla*

The first experiment was conducted to evaluate the effects of different melatonin concentrations and times of exposure on both species sperm motility for further application in cryopreservation experiment 2. Sperm samples were collected as describe above (section 2). For this experiment, six pools of sperm containing 10 to 12 Senegalese sole males per pool were made (n = 6) and nine sperm samples from individual European eel males (n = 9) were collected.

For both species, sperm samples were diluted in respective extender/cryoprotectants solutions according to previously described cryopreservation protocols for each species (Herranz-Jusdado et al., 2018; Riesco et al., 2017). For Senegalese sole, a dilution rate of 1:2 (sperm: extender) in Mounib extender solution (125 mM sucrose, 100 mM $KHCO_3$, 6.5 mM reduced glutathione), proposed by Chereguini et al. (2003), was used. The extender solution contained 10% DMSO and 10% EY as cryoprotectants (Riesco et al., 2017). For European eel, a dilution rate of 1:9 (sperm:extender) in P1 medium, proposed by Peñarada et al. (2010) was used. The extender solution contained 10% MeOH cryoprotectant (Herranz-Jusdado et al., 2018).

Melatonin supplemented solutions were prepared by dissolving melatonin (Sigma-Aldrich, M-5250) in each cryoprotectant solution and adding to extender to yield four different melatonin concentrations: 0.01, 0.1, 1 and 10 mM. In addition, an extender containing similar cryoprotectant concentration without melatonin was included as a control (10% DMSO and 10% EY or 10% MeOH). Sperm motility analysis of samples exposed to control and melatonin solutions was performed as previously described

(section 2). Four different exposure times for each species were experimented: 2, 5, 15 and 30 minutes for Senegalese sole and 5, 15, 30 and 60 minutes for European eel.

3.2 Experiment 2: effect of melatonin on F1 *Solea senegalensis* post-thaw sperm quality

The second experiment was conducted to evaluate the effect of melatonin supplementation in post-thaw *S. senegalensis* sperm relatively to the established protocol described by Riesco et al. (2017). Sperm samples were collected as previously described (section 2.1). For this experiment, eleven pools of sperm containing 10 to 14 males per pool were made (n = 11). For cryopreservation solution preparation, the same protocol described above in experiment 1 was followed (section 3.1). Considering the low sperm quantity of this species and in accordance with the motility results obtained previously in experiment 1, the two best melatonin concentrations were selected: 0.1 mM and 10 mM. An extender containing similar cryoprotectant concentration without melatonin was included as a control (10% DMSO and 10% EY).

Afterwards, sperm samples diluted in the respective solutions (i.e. control, 10 mM and 0.1 mM melatonin) were loaded into 0.25 mL straws and these were immediately placed on a horizontal rack 2 cm above liquid nitrogen in a Styrofoam box for 2 min of equilibration time. Consecutively, straws were frozen in nitrogen vapor for 10 min before plunging into liquid nitrogen and stored in a nitrogen container until thawing procedure. Sperm samples were thawed in a water bath at 25ºC for 15 s immediately before sperm analysis.

3.2.1. Motility analysis

Motility analysis of post-thaw sperm was performed as previously described (section 2.1). A total of 11 pools (n = 11) for each experimental group were analysed.

3.2.2. Viability analysis

Viability analysis was performed by combining post-thaw sperm samples with propidium iodide (PI) (Sigma-Aldrich) and measuring the sample fluorescence on a flow cytometer

(BD FACSCalibur™, BD Biosciences, CA). PI is a membrane impermeable dye that label cells with compromised membrane and emits red fluorescence. The settings for spermatozoa detection in the flow cytometer were previously adjusted by collecting total events as relation of forward scatter (FSC; cell size characterization) and side scatter (SSC; cell granularity) plots (Figure 1A). The gating (R1) of sperm population was used to exclude non-sperm events and it was plotted on the FSC and SSC profile of Senegalese sole post-thaw sperm. After PI staining, it was possible to detect a sperm population with high FL2 emission corresponding non-viable cells permeable to PI (Figure 1B).

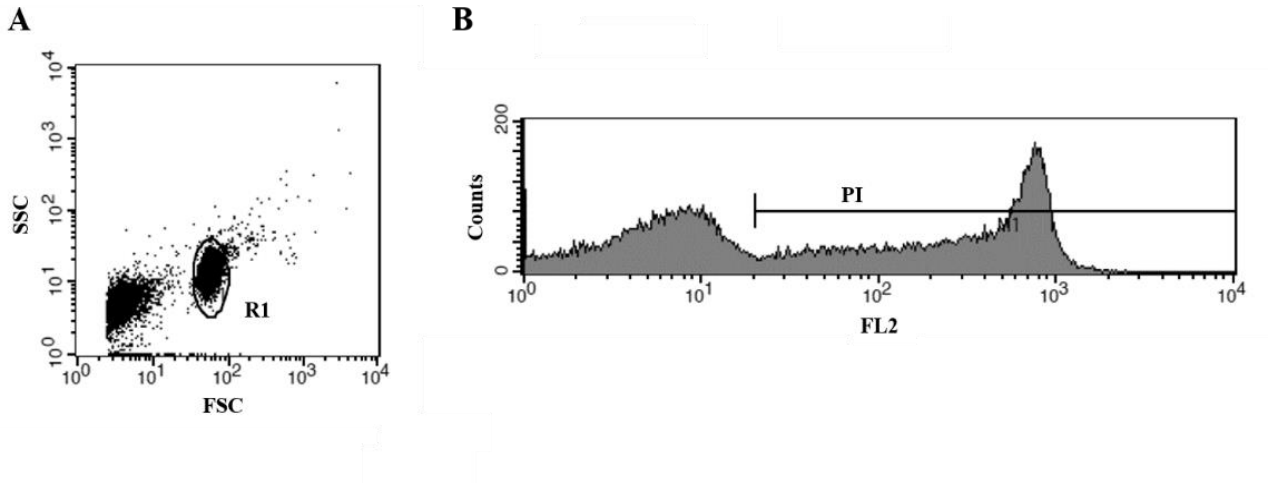


Figure 1 – Flow cytometry for viability analysis. **(A)** Dot plot cytograms showing sperm population (R1) gating (SSC vs FSC) and **(B)** single count histogram for PI. 0.1% cut off value was considered. FSC, forward scatter; SSC, size scatter, PI, propidium iodide, FL2, 585/42 nm filter.

Thereafter, for post-thaw sample analysis, 500 µL of 1% NaCl solution, 3 µL of thawed sperm and 1 µL of PI (1mg/mL) were added inside a cytometer tube. The samples were analysed after 5 min of incubation in the dark in a flow cytometer equipped with a 488 nm laser for PI detection with a 585/42 nm filter (FL2). A total of 30.000 events were counted for each sample and the percentage of non-viable cells was determined by BD CellQuest Pro software (version 8.7, BD Biosciences, CA). For viable cell determination, the percentage of unviable cells was subtracted from the percentage of sperm population. A total of 11 pools (n = 11) for each treatment were analysed.

3.2.3. DNA integrity analysis - Comet assay

Comet assay was performed to detect DNA fragmentation in spermatozoa, following the protocol described by Riesco et al. (2017). For sample preparation, 1 µL of thawed sperm was diluted in 300 µL of non-activating Ringer solution (40.23 mM KCl, 111.22 mM NaCl, 2.7 mM $CaCl_2$, 2.38 mM $NaHCO_3$). Afterwards, 20 µL of sperm mixture was diluted in 130 µL of agarose low melting point (0.5%) prepared previously in PBS. For slide preparation, 60 µL of agarose mixture were distributed into pre-coated slides with 0.5% normal agarose (dried overnight) and covered with a coverslip for 20 min at 4 ºC. The coverslip was removed and the slides were directly incubated in a copling jar containing lysis solution (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris pH 10, 1% Triton x100, 1% lauril sarcosine) for 1 h at 4 ºC. After lysis, the slides were placed horizontally in a electrophoresis cube (Sub-Cell GT, BioRad, Portugal) filled with previously prepared electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13). The electrophoresis was performed at 25 V and 300 mA for 10 min at 4 ºC. The volume of electrophoresis solution was adjusted to reach approximately 300 mA. Afterwards, the slides were washed twice with neutralizing solution (0.4 M Tris HCl, pH 7.5) for 5 min at 4 ºC, fixed in absolute ethanol during 3 min and kept in slide supports at 4 ºC until further observation.

For comet observation, 10 µL of diluted PI (20 µM) was placed in each sample and covered with a coverslip. The slides were observed in a fluorescence microscope (Nikon Eclipse E200) and images were captured and recorded with a digital camera (VisiCam 5 Plus, VWR). At least 100 cells per slide were scored and analysed using Kinetic Imaging Komet v6.0 software (Andor Technology, Ltd.). The percentage of tail DNA (% DNAt) was the parameter used to determine the amount of DNA fragmentation since it relates the amount and size of the DNA fragments. A total of 11 pools (n = 11) for each treatment were analysed.

3.2.4. Lipid Peroxidation – MDA assay

Lipid peroxidation was assessed by quantifying the concentration of malodialdehyde (MDA) through a colorimetric assay (kit BIOXYTECH LPO-586 ™, OxisResearch) and following the protocol described by Riesco et al. (2017). For sample preparation, thawed sperm was diluted in 300 µL of non-activating Ringer solution and centrifuged at 500 g

for 10 min at 10 ºC. After carefully removing the supernatant, the pellet was resuspended in 100 µL of Ringer solution and sonicated with 2 pulses for 5 s at 35% Amp. Afterwards, the sample was incubated in 10 µL of a solution containing 200 µM sodium ascorbate and 40 µM $FeSO_4$ for 30 min at 37 ºC in the dark. Meanwhile, a MDA calibration curve was prepared by diluting MDA standard solution (20 µM) in MiliQ (Table 1).

Table 1 – Curve points used in MDA protocol

Curve Points	**1**	**2**	**3**	**4**	**5**	**6**
MDA (µM)	10	8	4	2	1	0

After the incubation time, in eppendorf tubes containing 320 µL of diluted 1:3 (v/v) R1 solution in methanol, 100 µL of sample solution and curve point solutions were added. For blank point preparation, in an eppendorf containing 320 µL of diluted 1:3 (v/v) acetonitrile in methanol and, 100 µL of blank solution were added. Afterwards, 4 µL of probucol was added to all solutions (sample, blank and curve point solutions) and the eppendorf tubes were carefully vortexed. Subsequently, 75 µL of R2 reagent were added to each eppendorf tubes and then incubated for 1 hour at 45 ºC in the dark. After the incubation time, samples were centrifuged (10.000 g for 10 min at 4 ºC) and 200 µL of each supernatant were transferred to a 96-well flat-bottom transparent plate (Nunc). The absorbance was read in a microplate reader (Synergy 4, Biotek Instruments. Inc.) at 586 nm and MDA concentrations determined from standard curve calculation. The results were presented in nanomoles (nM) of MDA per million of spermatozoa. A total of 11 pools (n = 11) for each treatment were analysed.

3.2.5. ROS detection – DHE and SYTOX® green dyes

Reactive oxygen species (ROS) detection was performed by combining post-thaw sperm with dihydroethidium (DHE) and SYTOX® green (Invitrogen™, ThermoFisher) and measuring samples fluorescence in a flow cytometer (BD FACSCalibur™, BD Biosciences, CA). The DHE permeable dye detects superoxide ion production by staining the DNA in the nucleus upon oxidation and emitting red fluorescence. The SYTOX® green impermeable dye detects cells with compromised plasma membrane. The settings

for ROS detection by flow cytometry were previously adjusted by collecting total events as relation of forward scatter (FSC; cell size characterization) and side scatter (SSC; cell granularity) plots. The gating (R1) of sperm population was used to exclude non-sperm events and it was plotted on the FSC and SSC profile of post-thaw sperm (Figure 2A). For cytometer calibration, post-thawed sperm was incubated separately with each dye, to gate SYTOX® green (R2) (Figure 2B) and DHE (R8) (Figure 2C) labelled populations, and then in combination (SYTOX® green and DHE). This allowed the detection of four subpopulations corresponding to non-viable cells (DHE negative, SYTOX® green positive); non-viable cells producing ROS (DHE and SYTOX® green positive); viable cells producing ROS (DHE positive, SYTOX® green negative) and viable cells (DHE and SYTOX® green negative) (Figure 2D).

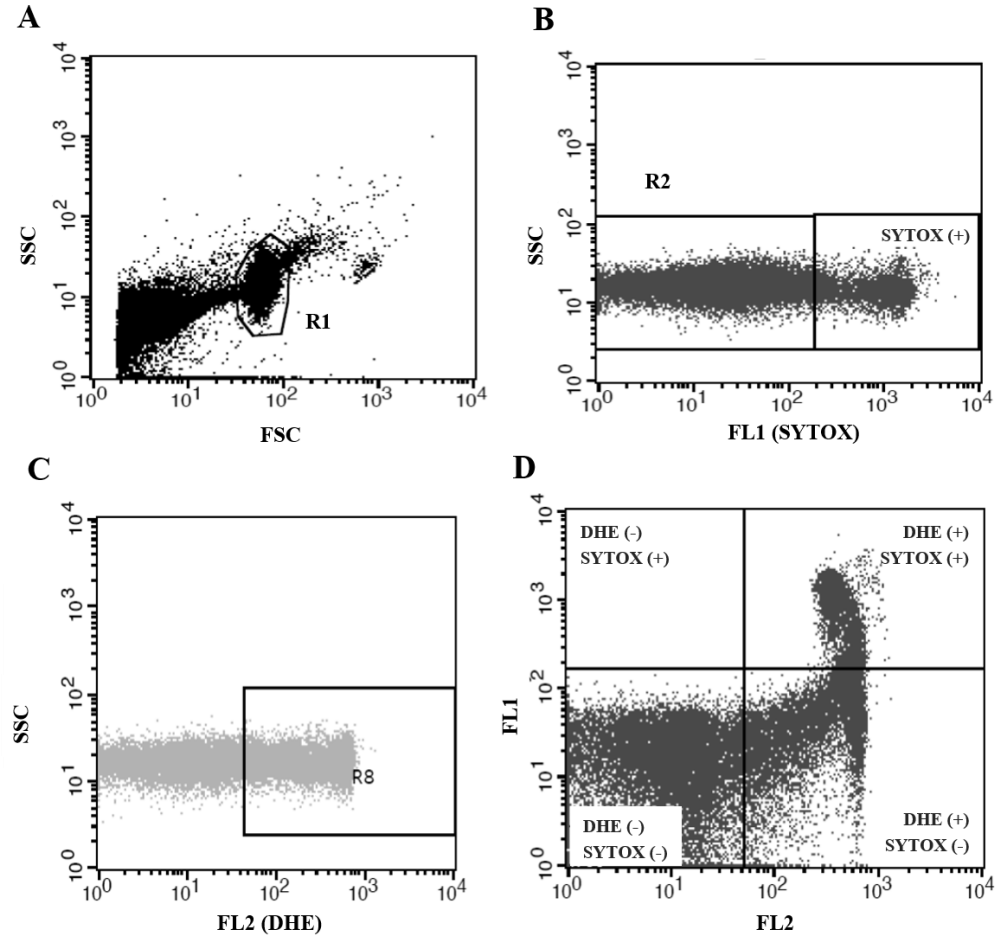


Figure 2 – Flow cytometry for ROS detection. (A) Dot plot cytograms showing sperm population (R1) gating (SSC vs FSC), (B) non-viable population (R2) labelled with SYTOX® green, (C) ROS producing population (R8) labelled with DHE and (D) four subpopulations corresponding to non-viable cells (DHE negative, SYTOX positive), non-viable cells producing ROS (DHE and SYTOX positive), viable cells producing ROS (DHE positive, SYTOX negative) and viable cells (DHE and SYTOX negative). 0.1% cut off value was considered. FSC, forward scatter; SSC, size scatter; DHE, dihydroethidium; SYTOX, SYTOX® green; FL1, 530/30 nm filter; FL2, 585/42 nm filter.

Thereafter, for sample analysis, 500 µL of 1% NaCl solution, 3 µL of thawed sperm and 0.5 µL of DHE (0.5 mM) were prepared inside a cytometry tube (BD Biosciences, CA). After 5 min of DHE incubation, 0.5 µL of SYTOX® green (1 µM) was added. The samples were analysed after 10 min of total incubation time in the dark in a flow cytometer equipped with a 488 nm laser for SYTOX® green detection with a 530/30 nm filter (FL1) and for DHE detection with a 585/40 nm filter (FL2). A total of 30.000 events were acquired for each sample and the percentage of non-viable, non-viable cells producing ROS, live cells producing ROS and live cells was determined by BD CellQuest Pro acquisition software (version 8.7, BD Biosciences, CA). A total of 11 pools (n=11) for each treatment were analysed.

3.2.6. Apoptosis detection – Caspase 3/7 and 7-AAD dyes

Caspase detection was performed by applying Muse™ Caspase-3/7 kit (Millipore), following kit instructions and adapting it for Senegalese sole post-thawed sperm. The Caspase-3/7 reagent and 7-aminoactinomycin D (7-AAD) were combined with post-thawed sperm and samples fluorescence was measured on a flow cytometer (BD FACSCalibur™, BD Biosciences, CA). The Muse Caspase-3/7 reagent is cell membrane permeable and contains a specific peptide (DEVD) conjugated to a DNA binding dye. The cleavage of DEVD substrate by active caspase 3/7 in apoptotic cells results in release of the DNA dye to the nucleus and green fluorescence emission, thus labelling the apoptotic cells. The 7-AAD permeates cells with compromised membrane, thus leaving the viable cell unstained. The settings were previously adjusted by collecting total events as relation of forward scatter (FSC; cell size characterization) and side scatter (SSC; cell granularity) plots. The gating (R1) of sperm population was used to exclude non-sperm events and it was plotted on the FSC and SSC profile of post-thaw sperm (Figure 3A). For cytometer calibration, post-thawed sperm was incubated separately with each dye, to gate Caspase 3/7 (R2) (Figure 3B) and 7-AAD (R3) (Figure 3C) labelled populations, and then in combination (Caspase3/7 and 7-AAD). This allowed the detection of four subpopulations corresponding to necrotic cells dead by other mechanisms unrelated to apoptosis (Caspase-3/7 negative,7-AAD positive); late apoptotic cells (Caspase-3/7 and 7-AAD positive); early apoptotic cells exhibiting caspase 3/7 activity (Caspase-3/7

positive,7-AAD negative) and viable cells (Caspase-3/7 and 7-AAD negative) (Figure 3D).

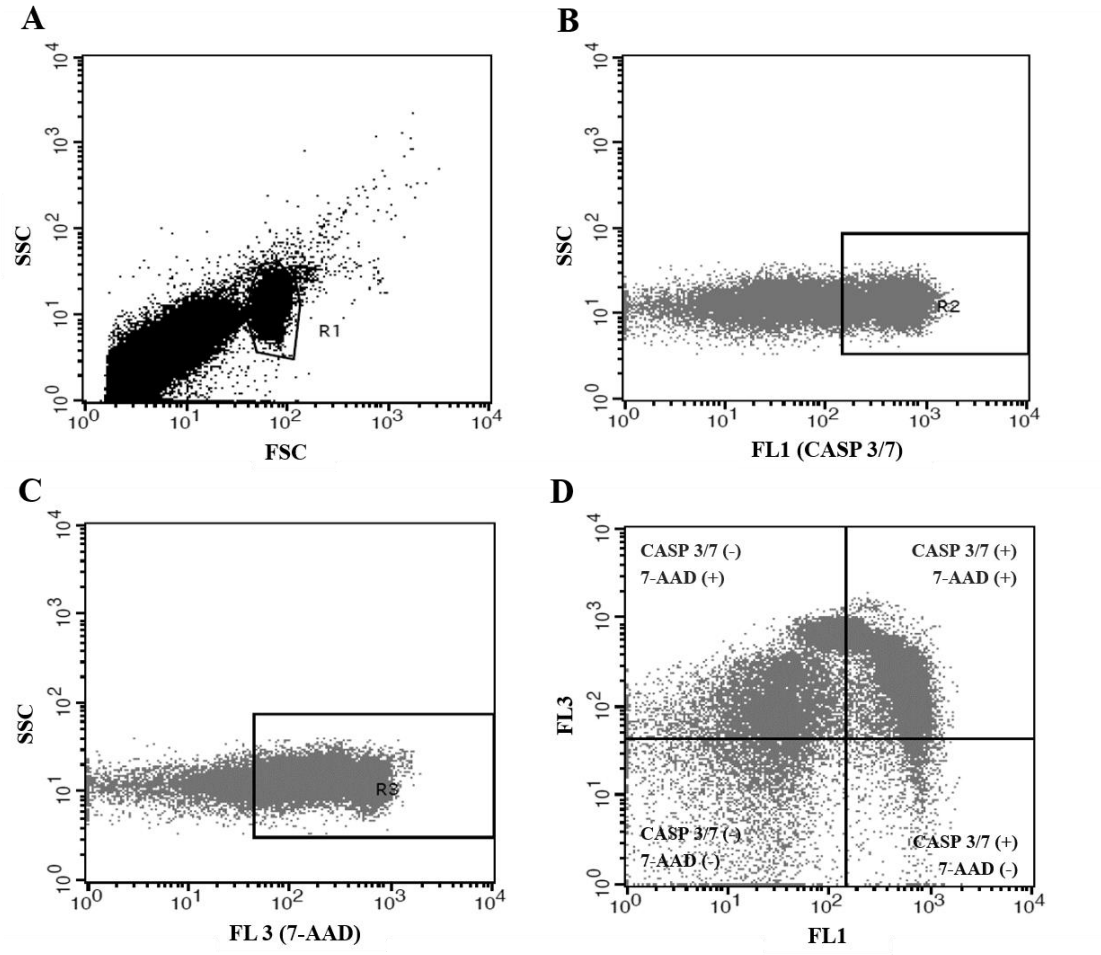


Figure 3 – Flow cytometry for apoptotic cells detection. **(A)** Dot plot cytograms showing sperm population (R1) gating (SSC vs FSC), **(B)** apoptotic cell population (R2) labelled with Caspase-3/7, **(C)** dead cell population (R3) labelled with 7-AAD and **(D)** four populations corresponding to necrotic cells (CASP 3/7 negative, 7-AAD positive), late apoptotic cells (CASP 3/7 and 7-AAD positive), early apoptotic cells (CASP 3/7 positive, 7-AAD negative) and viable cells (CASP 3/7 and 7-AAD negative). 0.1% cut off value was considered. FSC, forward scatter; SSC, size scatter; CASP 3/7, Caspase-3/7; 7-AAD, 7-aminoactinomycin D; FL1, 530/30 nm filter; FL3, 670 nm long pass filter.

Thereafter, for sperm analysis, 250 µL of 1% NaCl solution, 3 µL of post-thawed sperm and 5 µL of Caspase-3/7 diluted 1:8 (v/v) in PBS were prepared inside a cytometry tube (BD Biosciences, CA). After 30 min of Caspase-3/7 incubation, 150 µL of 7-ADD diluted 1:36 (v/v) in kit included buffer was added. The samples were analysed after 35 min of total incubation in the dark in a flow cytometer equipped with a 488 nm laser for Caspase-3/7 detection with a 530/30 nm filter (FL1) and for 7-AAD detection with a 670 nm long pass filter (FL3). A total of 30.000 events were acquired for each sample and the

percentage of necrotic cells dead by other mechanisms, late apoptotic, early apoptotic, and viable cells was determined by BD CellQuest Pro acquisition software. A total of 11 pools (n=11) for each treatment were analysed.

4. Statistical analysis

IBM SPSS Statistics version 26.0 software (IBM, USA) was used for statistical analysis. Data were expressed as means and normalized by logarithmic, or arcsine transformation when results were expressed as percentages. The verification of homogeneity of variances was performed using Levene's test. The considered level of significance was 0.05 ($p < 0.05$).

In experiment 1, a two-way ANOVA followed by a Student-Newman-Keuls (SNK) *post-hoc* was used to evaluate the effects of different melatonin concentrations throughout different exposure times in sperm motility of *S. senegalensis* and *A. anguilla*. In experiment 2, a general linear model with a Bonferroni correction was performed to compare the effects of melatonin concentrations on post-thaw sperm motility of *S. senegalensis*. A one-way ANOVA followed by SNK *post-hoc* was performed to compare the different treatments in terms of viability, DNA fragmentation, MDA quantification, ROS, and caspase 3/7 detection in post-thaw sperm.

Results

1. Experiment 1: effect of exposure time and melatonin concentrations in sperm motility of Senegalese sole (*S. senegalensis*) and European eel (*A. anguilla*)

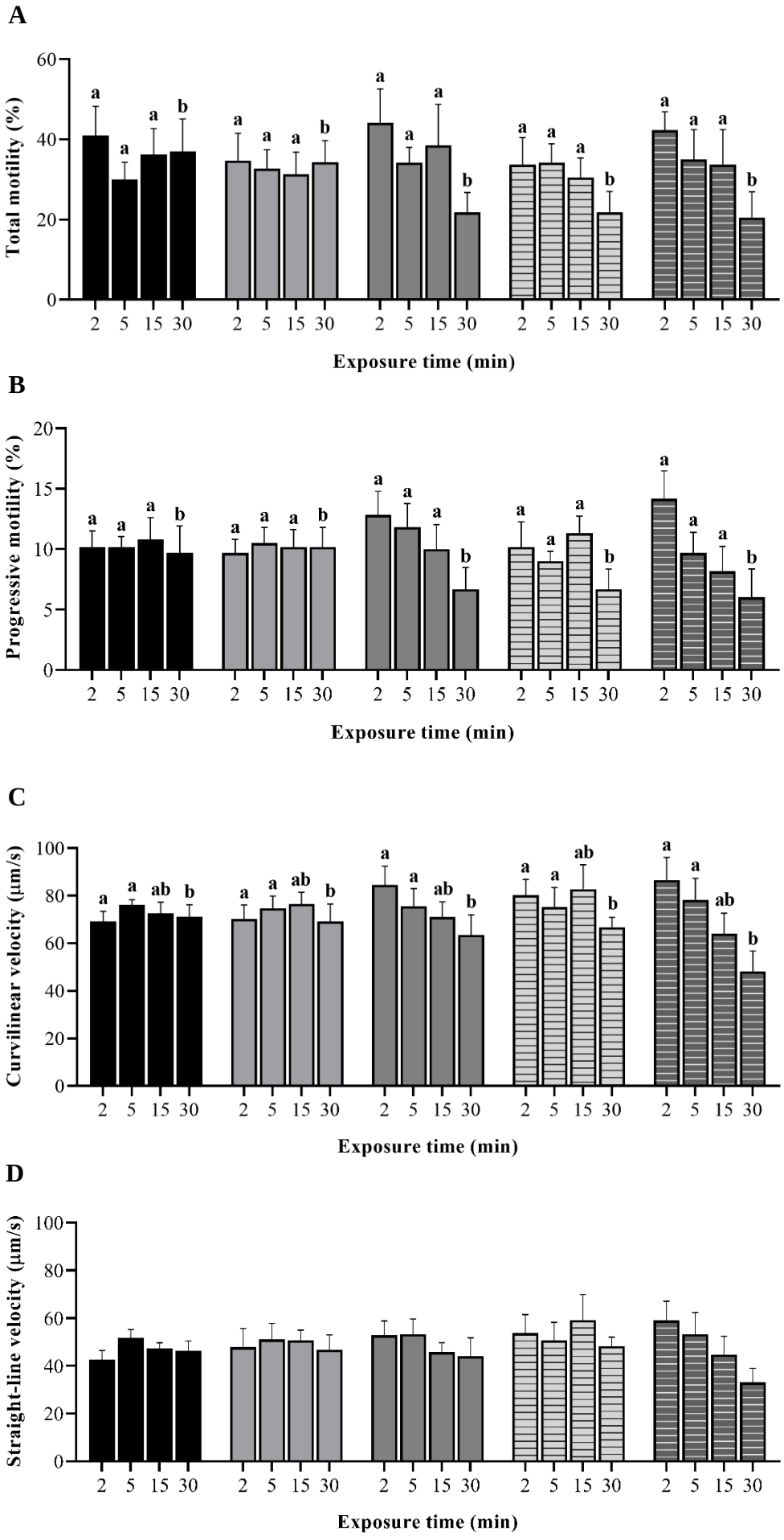
1.1 Senegalese sole

There were no interactions between exposure time and melatonin concentration effects in any sperm motility descriptors and each variable could be analysed independently. Exposure time caused the largest effect, specifically in total and progressive motility, and curvilinear velocity. The melatonin concentration did not significantly affect any sperm descriptors. For sperm motility analysis, a two-way ANOVA (SNK, $p < 0.05$) was performed to understand the effects of different exposure times, melatonin concentrations and interactions for sperm total (TM) and progressive (PM) motility, curvilinear (VCL) and straight-line (VSL) velocity, and linearity (Table 2).

Table 2 – *S. senegalensis* sperm motility at 15 seconds post-activation related to the effect of exposure time, melatonin concentration and their interactions for sperm total (TM) and progressive (PM) motility, curvilinear (VCL) and straight-line (VSL) velocity, and linearity (LIN). Statistical differences are in bold and were detected by a two-way ANOVA followed by a SNK as a *post-hoc* test to compare between variables within each sperm descriptor ($p < 0.05$).

	TM	**PM**	**VCL**	**VSL**	**LIN**
Exposure Time (min)	**0.026**	**0.004**	**0.029**	0.090	0.532
Melatonin Concentration (mM)	0.743	0.720	0.422	0.688	0.711
Exposure Time x Concentration	0.788	0.432	0.202	0.561	0.990

In sperm motility descriptors with significant differences, the results obtained for total (Figure 4A) and progressive (Figure 4B) motility and for curvilinear velocity (Figure 4C) at 2-min exposure time were significantly higher than at 30-min exposure time within different concentrations. Regarding the 2-min exposure time, sperm motility with 0.1 mM melatonin (TM – 44.1 ± 8.4%, PM – 12.8 ± 1.95%, VCL – 84.5 ± 6.7 μm/s) and 10 mM melatonin (TM – 42.3 ± 4.6%, PM – 14.1 ± 2.3%, VCL – 86.5 ± 9.6 μm/s) increased in comparison to control (TM – 41 ± 7.27%, PM – 10.1 ± 1.32%, VCL – 69 ± 4.11 μm/s) although no significant differences were found between melatonin concentrations. No significant differences were revealed in straight-line velocity (Figure 4D) and linearity (Figure 4E).


A
Total motility (%)
Exposure time (min)
B
Progressive motility (%)
Exposure time (min)
C
Curvilinear velocity (μm/s)
Exposure time (min)
D
Straight-line velocity (μm/s)
Exposure time (min)

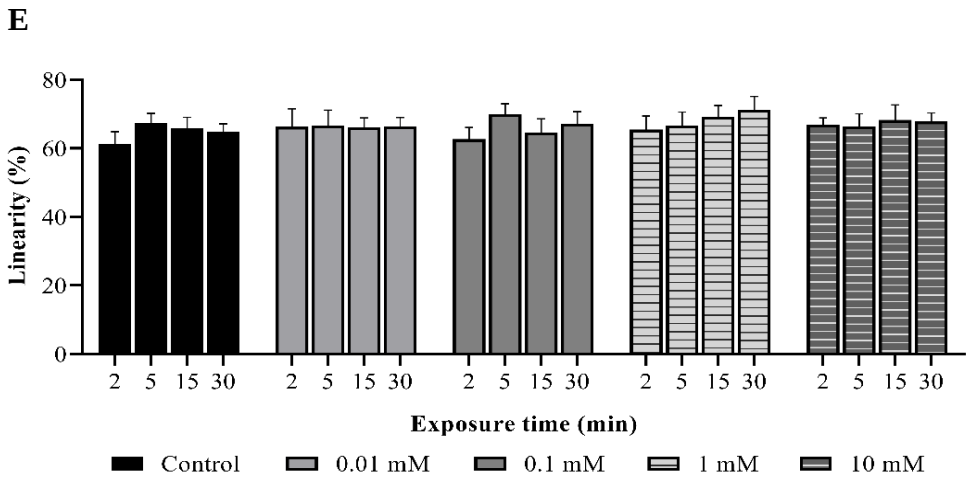


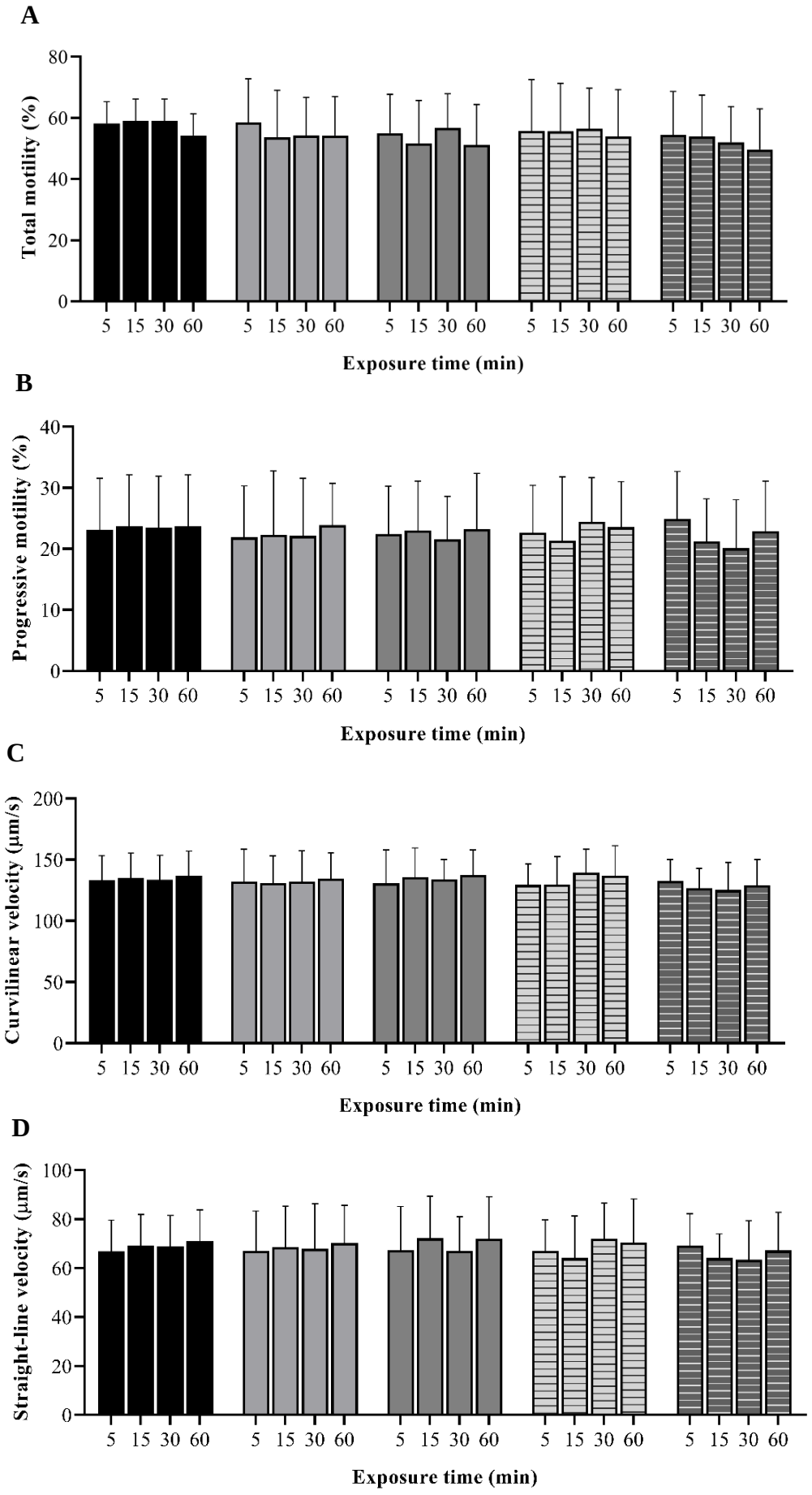
Figure 4 – *S. senegalensis* sperm motility at 15 s post-activation with different melatonin concentrations (control, 0.01 mM, 0.1 mM, 1 mM, and 10 mM) at different exposure times (2, 5, 15 and 30 minutes). Sperm motility was analysed for **(A)** total and **(B)** progressive motility, **(C)** curvilinear and **(D)** straight-line velocity, and **(E)** linearity. Data are expressed as a mean ± standard error of 6 pools for each treatment (n = 6). Statistical analysis was performed using a two-away ANOVA with a SNK (Student-Newman-Keuls) as *post-hoc* test to compare different treatments ($p < 0.05$). Different letters refer to significant differences between the different exposure time within the same concentration.

1.2 European eel

The results showed that there were no interactions between exposure time and melatonin concentration effects in any sperm motility descriptors and each variable could be analysed independently. Both melatonin concentration and exposure time did not significantly affect any sperm motility descriptors (Figure 5). For sperm motility analysis, a two-way ANOVA (SNK, $p < 0.05$) was performed to understand the effects of different exposure times, melatonin concentrations and interactions in sperm total and progressive motility, curvilinear and straight-line velocity, and linearity (Table 3).

Table 3 – *A. anguilla* sperm motility at 15 seconds post-activation related to the effect of exposure time, melatonin concentration and their interactions for sperm total (TM) and progressive (PM) motility, curvilinear (VCL) and straight-line (VSL) velocity, and linearity (LIN). Statistical differences are in bold and were detected by a two-way ANOVA followed by a SNK as a *post-hoc* test to compare between variables within each sperm descriptor ($p < 0.05$).

	TM	**PM**	**VCL**	**VSL**	**LIN**
Exposure Time (min)	0.258	0.801	0.418	0.411	0.674
Melatonin Concentration (mM)	0.967	0.971	0.987	0.992	0.954
Exposure Time x Concentration	0.995	0.942	0.902	0.953	0.982


A
Total motility (%)
0
20
40
60
80
5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60
Exposure time (min)
B
Progressive motility (%)
0
10
20
30
40
5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60
Exposure time (min)
C
Curvilinear velocity (µm/s)
0
50
100
150
200
5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60
Exposure time (min)
D
Straight-line velocity (µm/s)
0
20
40
60
80
100
5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60 5 15 30 60
Exposure time (min)

E

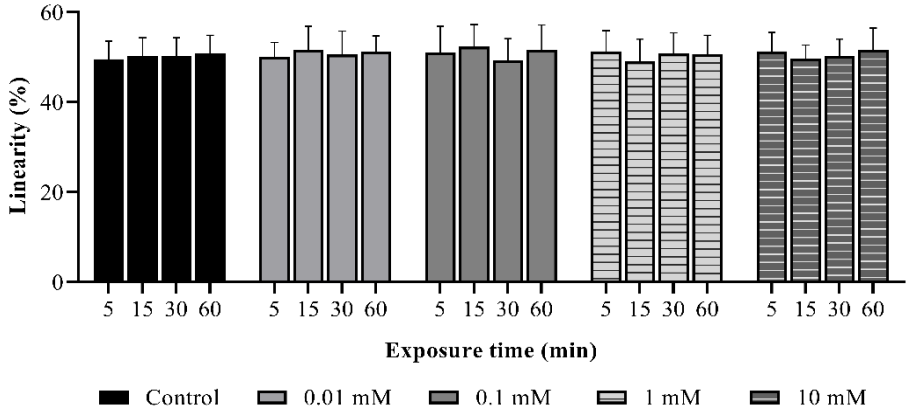


Figure 5 – *A. anguilla* sperm motility at 15 s post-activation with different melatonin concentrations (control, 0.01 mM, 0.1 mM, 1 mM, and 10 mM) at different exposure times (5, 15, 30 and 60 minutes). Sperm motility was analysed for **(A)** total and **(B)** progressive motility, **(C)** curvilinear and **(D)** straight-line velocity, and **(E)** linearity. Data are expressed as a mean ± standard deviation of 9 males for each treatment (n = 9). Statistical analysis was performed using a two-away ANOVA with a SNK (Student-Newman-Keuls) as *post-hoc* test to compare different treatments ($p < 0.05$).

2. Experiment 2: effect of melatonin in F1 *Solea senegalensis* post-thaw sperm quality

2.1 Motility analysis

For motility analysis, a general linear model was performed to evaluate the efficiency of melatonin supplementation in post-thawed sperm motility. Throughout post-activation time, the total (TM) and progressive (PM) motility, curvilinear (VCL) and straight-line (VSL) velocity, and linearity (LIN) decreased considerably (Figure 6). Although there were no significant differences between experimental conditions at 15 s post-activation, post-thawed sperm with a 10 mM melatonin supplement presented higher sperm curvilinear and straight-line velocity, and linearity (VCL - 57 ± 2.75 μm/s, VSL - 45 ± 2.58 μm/s, LIN - 80 ± 4 %) in comparison to the control (VCL - 45 ± 3 μm/s, VSL - 35 ± 2 μm/s, LIN - 76 ± 3 %).

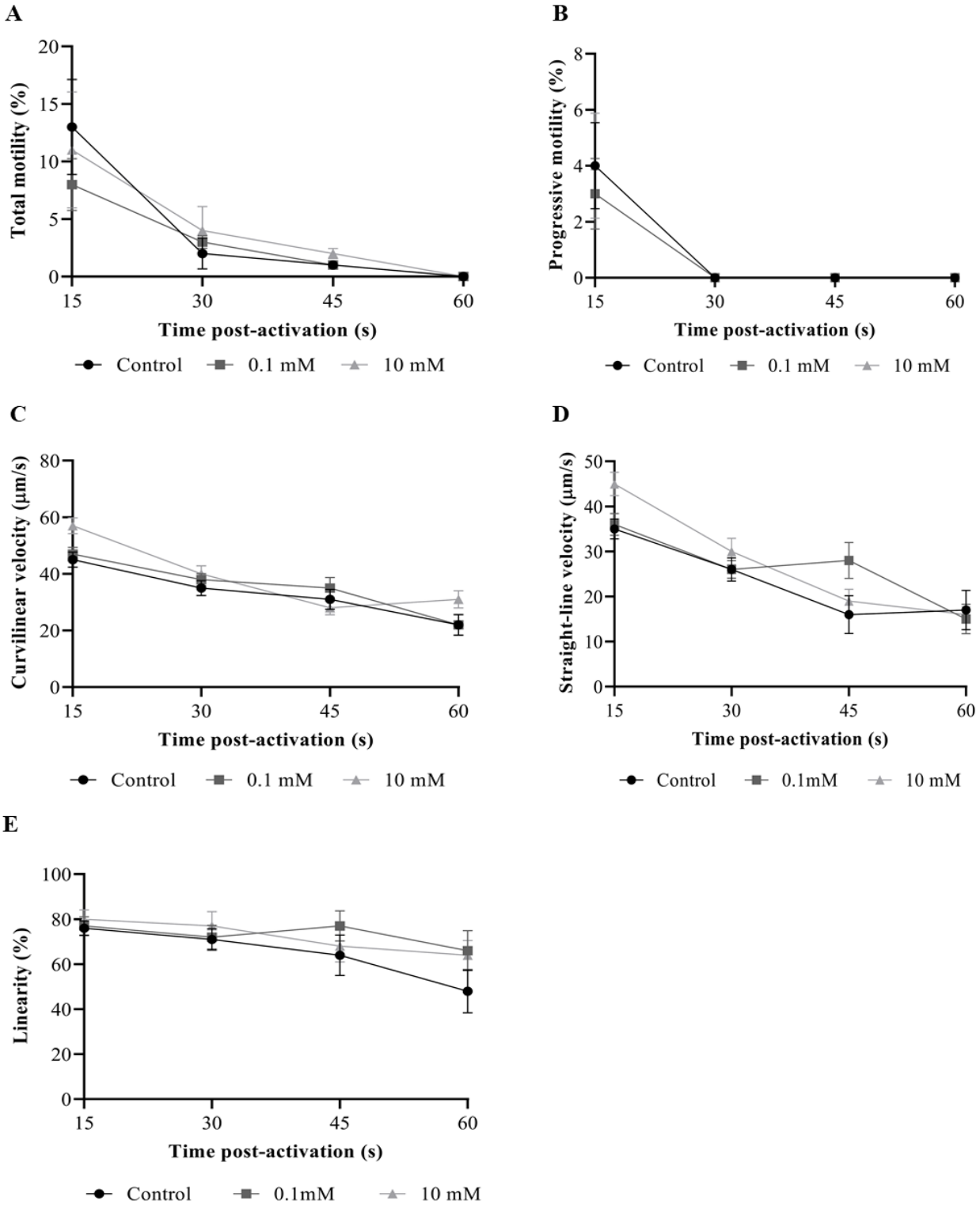


Figure 6 – *S. senegalensis* sperm **(A)** total and **(B)** progressive motility, **(C)** curvilinear and **(D)** straight-line velocity and **(D)** linearity at 15, 30, 45 and 60 seconds post-activation under different experimental conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean ± standard error of 11 pools for each experimental condition (n = 11). Statistical analysis was performed using a general linear model with a Bonferroni correction to compare different conditions ($p < 0.05$).

2.2 Viability analysis

The viability results obtained using PI showed viability percentages higher than 40% in all experimental conditions (Figure 7). The viability was higher in post-thawed sperm with 0.1 mM melatonin supplement (62.31 ± 9.11%) when compared with control (57.99 ± 11.21%), although no significant differences were found. However, post-thawed sperm with 10 mM melatonin supplement (43.65 ± 10.60%) was significantly lower when compared to the control.

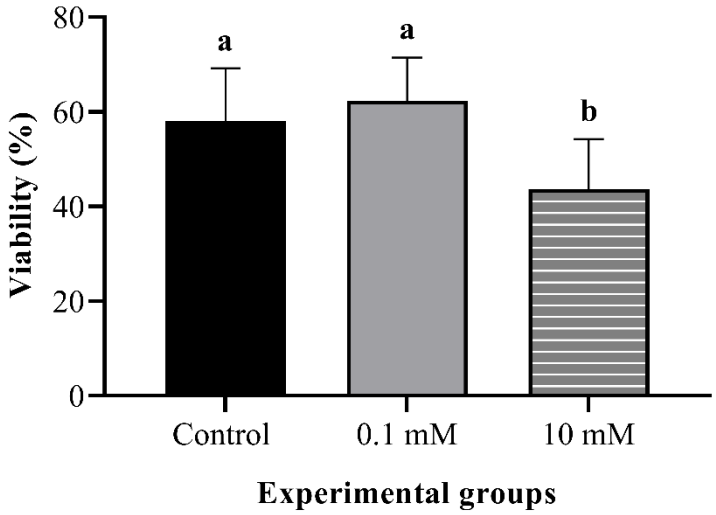


Figure 7 – Percentage of viable cells in *S. senegalensis* post-thawed sperm under different experimental conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean of percentages ± standard deviation of 11 pools for each experimental condition (n = 11). Statistical analysis was performed using one-way ANOVA followed by a Student-Newman-Keuls (SNK) as a *post-hoc* test to compare different conditions ($p < 0.05$). Different letters refer to significant differences.

2.3 DNA integrity analysis – Comet assay

The DNA fragmentation rates provided as percentage of DNA in the tail (DNAt) were lower than 12% in all experimental groups (Figure 8). The percentage of DNA fragmentation was lower in post-thawed sperm with 0.1 mM melatonin (10.98 ± 4.59%) and 10 mM melatonin (11.76 ± 5.77%) supplement when compared with control (11.82 ± 3.66%), although no significant differences were found between experimental conditions.

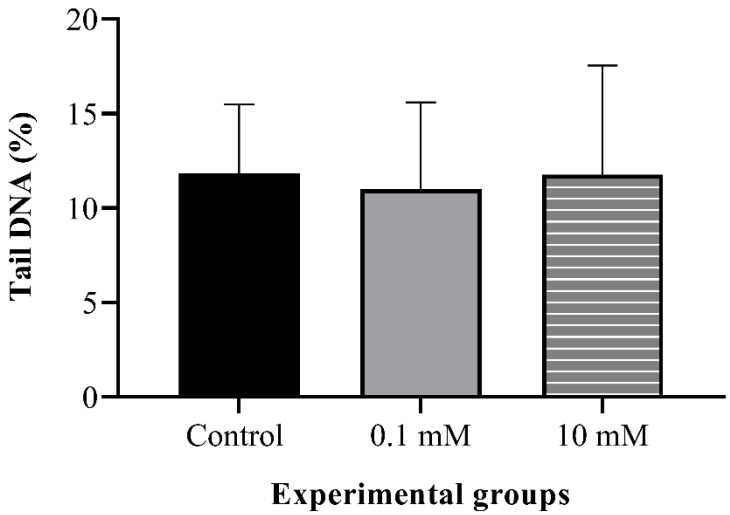


Figure 8 – Percentage of DNA fragmentation in *S. senegalensis* post-thawed sperm under different experimental conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean of percentages ± standard deviation of 11 pools for each experimental condition (n = 11). Statistical analysis was performed using one-way ANOVA followed by a Student-Newman-Keuls (SNK) as a *post-hoc* test to compare different conditions ($p < 0.05$).

2.4 Lipid Peroxidation – MDA assay

The quantification of MDA was determined in all post-thawed sperm as a measure of lipid peroxidation status (Figure 9).

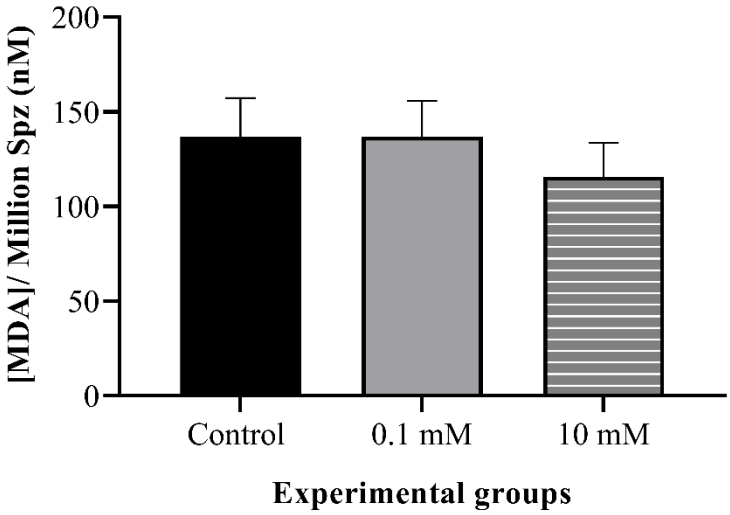


Figure 9 – Lipid peroxidation (MDA concentration) per million spermatozoa (nM) in *S. senegalensis* post-thawed sperm under different experimental conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean of percentages ± standard deviation of 11 pools for each condition (n = 11). Statistical analysis was performed using one-way ANOVA followed by a Student-Newman-Keuls (SNK) as a *post-hoc* test to compare different conditions ($p < 0.05$).

The lowest MDA concentration was found in post-thawed sperm with 10 mM melatonin (115.64 ± 17.90 nmoles of MDA per million spermatozoa) and 0.1 mM melatonin (136.76 ± 18.6 nmoles of MDA per million spermatozoa) supplement comparatively to control (136.86 ± 20.40 nmoles of MDA per million spermatozoa), although no significant differences were found between experimental conditions.

2.5 ROS detection – DHE and SYTOX® green dyes

The detection of reactive oxygen species (ROS) in post-thaw sperm was performed by DHE and SYTOX® green dyes. Although four sperm cell populations were established as described above (section 3.2.5), the most relevant for post-thawed sperm analysis were the viable and viable producing ROS cell populations (Figure 10). Regarding the viable cells population, post-thawed sperm with 0.1 mM melatonin (42.56 ± 10.57%) and 10 mM melatonin (36.55 ± 12.26%) supplement exhibited low percentages in comparison with the control (43.65 ± 11.21%). Consequently, the percentage of viable cells producing ROS was highest with 10 mM (40.88 ± 9.81%) and 0.1 mM (37.63 ± 5.49%) melatonin supplement when compared with the control (36.66 ± 6.42%). However, there were no significant differences between experimental conditions within post-thawed sperm populations.

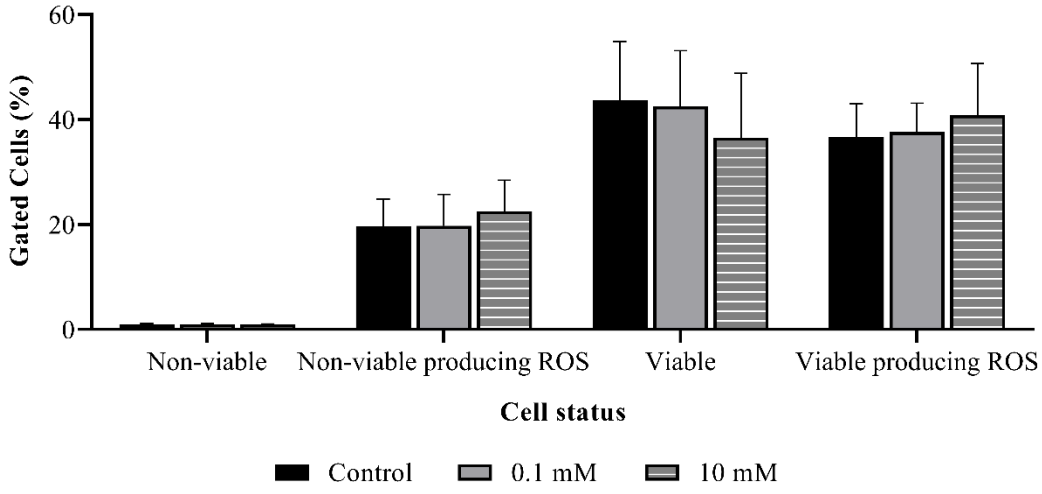


Figure 10 – Reactive oxygen species (ROS) detection in *S. senegalensis* post-thawed sperm under different experimental conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean of percentages ± standard deviation of non-viable, non-viable producing ROS, viable, and viable producing ROS cells of 11 pools for each experimental condition (n = 11). Statistical analysis was performed using one-way ANOVA followed by a Student-Newman-Keuls (SNK) as a *post-hoc* test to compare different experimental conditions ($p < 0.05$).

2.6 Apoptosis detection – Caspase 3/7 and 7-AAD dyes

The detection of apoptosis in post-thawed sperm was performed by Caspase-3/7 and 7-AAD dyes (Figure 11). Although four sperm populations were established as described above (section 3.2.6), the most relevant for post-thawed sperm analysis were the necrotic cells, late apoptotic cells, and early apoptotic cells population. Among the populations of necrotic cells, post-thawed sperm with 0.1 mM melatonin (25.20 ± 7.11%) and 10 mM melatonin (22.95 ± 6.24%) supplement exhibited lower percentages in comparison with the control (27.58 ± 7.15%), although there were no significant differences. In the late apoptotic cells population, the percentage in post-thawed sperm with 10 mM supplementation (58.92 ± 6.24%) was significantly higher than control (53.66 ± 5.46%). However, the percentages in post-thawed sperm with 0.1 mM melatonin supplement (54.74% ± 4.08%) did not show significant differences. In the early apoptotic cells population, post-thawed sperm with 0.1 mM melatonin (7.22 ± 3.21%) and 10 mM melatonin (7.01 ± 2.55%) supplementation revealed higher caspase 3/7 activity when compared to the control (6.76 ± 2.28%).

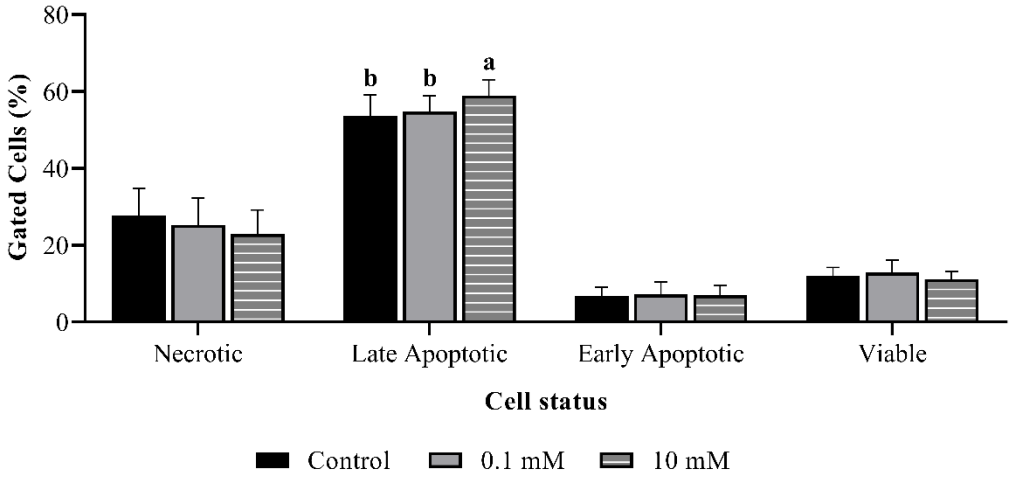


Figure 11 – Caspase detection by flow cytometer in *S. senegalensis* thawed sperm under different treatment conditions (control, 0.1 mM, and 10 mM melatonin). Data are expressed as a mean of percentages ± standard deviation of necrotic cells, late apoptotic, early apoptotic, and viable cells of 11 pools for each experimental condition (n = 11). Statistical analysis was performed using one-way ANOVA followed by a Student-Newman-Keuls (SNK) as a *post-hoc* test to compare different experimental conditions ($p < 0.05$). Different letters refer to significant differences.

Discussion

1. Effect of exposure time and melatonin concentrations in fish sperm motility

Melatonin is an ubiquitous molecule present among all evolutionary life forms, from multicellular to unicellular organisms (Zhao et al., 2019). Melatonin is widely known for its importance in many physiological functions such as circadian rhythms synchrony, which include reproductive cycle control. However, this molecule achieves a remarkable multifunctionality and it contributes to a variety of cellular mechanisms which contribute for cell homeostasis regulation and oxidative damage protection (Gurer-Orhab and Suzen, 2015). There is evidence that melatonin can target directly cell metabolism through various molecular pathways, which ultimately lead to cell progressive adaptation (Liu et al., 2019). Thus, the effect of melatonin in spermatozoa biology has drawn increasing attention, as spermatozoa are structurally susceptible to oxidative damage through lipid peroxidation and DNA oxidation (Aitken et al., 2012; Cebrián-Pérez et al., 2014). In other vertebrates, melatonin supplement promoted sperm functionality by increasing motility and, overall sperm performance (Fujinoki, 2008; Gonzalez-Arto et al., 2016). However, knowledge concerning melatonin effect in sperm remains limited in many species, including fish. In particular, fish spermatozoa could benefit greatly from melatonin supplementation as these possess a high PUFAs membrane content, which is especially a good target for oxidative stress damage (Cabrita et al., 2014).

In this perspective, the first experiment was performed to evaluate the effect of melatonin supplement on fish sperm motility. Furthermore, sperm motility mechanisms in which melatonin can take part might be elucidated, as there are no studies concerning this topic in fish. Therefore, F1 Senegalese sole and European eel sperm motility was evaluated at 15 seconds post-activation after being exposed to different melatonin concentrations during various exposure times. Regarding F1 Senegalese sole, the exposure time to melatonin supplemented solution significantly reduced sperm motility throughout melatonin incubation time. This might be associated with the rapid decrease of sperm quality throughout time, which is reflected in sperm motility decline. Moreover, although DMSO is considered one of the most effective cryoprotectants for most fish species, it can also produce toxic effects (Cabrita et al., 2010b) and the results obtained from this experiment corroborate it with sperm motility reduction throughout of time exposure.

Regarding the exposure time effect, in European eel, there were no significant differences between experimental groups. This may be explained by the species spermatozoa tolerance to methanol (10% MeOH), respectively used to dissolve the melatonin powder. Other studies performed in European eel sperm cryopreservation indicated that MeOH does not affect the extender osmolality, relatively similar to P1 medium (Szabó et al., 2005).

Concerning melatonin concentration effects, the results obtained from this experiment revealed that melatonin supplement at the considered concentration range (0.01 to 10 mM) did not significantly influence sperm motility in both species. In F1 Senegalese sole sperm, the motility values registered in this experiment for the control group (i.e. without melatonin) were lower than those described for F1 breeders of this species in other studies (Riesco et al., 2019). Nevertheless, Riesco et al. (2019) analysed sperm samples from individual breeders, whereas sperm samples from this experiment, were gathered from several males to generate sperm pools. Sperm pools usually present inferior sperm motility (Cabrita, personal communication) and, therefore sperm quality may be affected. However, at 2-minute exposure with 0.1 mM and 10 mM melatonin supplement, F1 Senegalese sole sperm exhibited some improvement in motility comparatively with the control, specifically in total and progressive motility, and curvilinear velocity. In the European eel, sperm motility of supplemented samples revealed similar values to those recorded in sperm samples without melatonin (control). Furthermore, sperm motility rates from control group samples were consistent with those described for this species, which corroborates the hypothesis that melatonin supplement at these concentrations had a minor effect in European eel spermatozoa motility (Caldeira et al., 2019; Gallego et al., 2014).

From a general point of view, these results are consistent with other studies performed in vertebrates, which have found that melatonin effect can be different between species and melatonin concentrations (Fernández-Alegre et al., 2020; Gimeno-Martos et al., 2019). For instance, previous studies have demonstrated that incubation with melatonin at millimolar concentration range improved human (Najafi et al., 2018) and bull (Ashrafi et al., 2013) sperm motility. Similarly, melatonin incubation with pico to nanomolar range promoted sperm hyperactivation in hamster (Fujinoki, 2008). The addition of melatonin at same concentration range used in hamster sperm revealed a motility increase in rapid spermatozoa subpopulations in ram sperm. However, in the same study, melatonin

supplementation with micromolar concentration did not improve sperm motility and prevented the increase of capacitated spermatozoa (Gimeno-Martos et al., 2019). The same inhibitory effect was observed in spermatozoa subpopulations motility from bull with picomolar melatonin concentrations (Fernández-Alegre et al., 2020). In these studies, melatonin adverse effect on sperm motility was supported by intracellular cyclic adenosine monophosphate (cAMP) level decrease (Fernández-Alegre et al., 2020; Gimeno-Martos et al., 2019). Altogether, the different effects of melatonin reported among species suggests distinct melatonin cellular pathways. It is possible that melatonin can act through specific membrane receptors, which have been widely described in several species spermatozoa as G protein-coupled *mt1* (*mel1a*) and *mt2* (*mel1b*) receptors (Gonzalez-Arto et al., 2016). These membrane receptors interact with secondary messengers, such as the enzyme adenylyl cyclase (AC) and cAMP, which are fundamental mediators in sperm motility (Buffone et al., 2015). It has been described in melanophores that melatonin binding to these membrane receptors leads to an inhibitory effect coupled with AC activity decrease and consequent intracellular cAMP reduction (Andersson et al., 2003). Thus, this cascade would certainly result in spermatozoa motility decrease, and therefore it is indeed possible that this receptor mediated mechanism might be involved in the inhibitory melatonin effect observed in ram (Gimeno-Martos et al., 2019) and bull (Fernández-Alegre et al., 2020), where these receptors have been described (González-Arto et al., 2017; Li et al., 2019). Interestingly, Zilli et al. (2017) defined the same secondary messengers in seawater fish spermatozoa and revealed that the activation of AC leads to cAMP protein phosphorylation/dephosphorylation and subsequent sperm motility initiation (Zilli et al., 2017). Therefore, it is questionable if melatonin effect seen in F1 Senegalese sole is truly mediated through melatonin specific membrane receptors. Moreover, it is possible that such membrane receptors may be absent in this fish species spermatozoa. For instance, in stallion spermatozoa, *mt1* and *mt2* were not identified (Balao Da Silva et al., 2011). Besides, this suggestion is in accordance with results obtained from previous studies performed by our group, in which qPCR analysis revealed that *mt2* receptor expression in Senegalese sole spermatozoa were low, in both poor and high sperm quality (Morini et al., 2019).

Interestingly, the F1 Senegalese sole sperm motility increased slightly with melatonin and this may indicate melatonin ability to freely cross the cell membrane, therefore sustaining a receptor-independent melatonin activity. This has been widely proposed to explain

melatonin potential to access subcellular compartments, which can generate further independently or by secondary interaction with components of receptor-dependent pathways (Luchetti et al., 2010). In fact, melatonin uptake into cells and eventual access to essential subcellular components, such as mitochondria, is assumed to be related with melatonin lipophilic nature. This, in turn, would allow melatonin to diffuse through lipid-rich cell membrane with consequent entrance through mitochondrial membranes (Reiter et al., 2017). In fact, mitochondria have been referenced as a major target for melatonin, once it can increase mitochondrial membrane potential, therefore leading to ATP production coupled with inhibition of pro-apoptotic factors and, apoptosis prevention (Fang et al., 2020). Considering fish spermatozoa structure, melatonin could have access to subcellular compartments, as these sperm cells have a lipidic membrane with high PUFAs content (Cabrita et al., 2014) and, most importantly, are packed with mitochondria for energetic generation crucial to sustain energy demanding motility (Cosson, 2019). Therefore, in further studies, melatonin effect at the mitochondria potential membrane for energic production linked with its antioxidant potential against oxidative stress damage should be further assessed in fish spermatozoa. Another possible melatonin action could be through its performance as a phosphodiesterase inhibitor in similarity to other stimulatory components described in fish sperm motility, such as caffeine. For instance, caffeine has been portrayed as a phosphodiesterase inhibitor that can increase the levels of cAMP derived from ATP, thus stimulating protein phosphorylation and, consequently sperm motility in marine species (Suquet et al., 2012). In fact, caffeine supplement in dilution medium improved sperm motility in fish species (Carvalho et al., 2014). Although information regarding this same potential effect of melatonin is limited in spermatozoa, the same inhibitory mechanism through phosphodiesterase inhibition has been demonstrated in other cell types (Benítez-King et al., 2005; Huerto-Delgadillo et al., 1994). Similarly, melatonin may also act through calcium/calmodulin (Ca^{2+}/CaM) depend-protein phosphorylation inhibition. Such potential has been proposed in other cells by Huerto-Delgadillo et al. (1994), which stated that melatonin strongly interacts with calmodulin (CaM), therefore inhibiting CaM phosphodiesterase (Huerto-Delgadillo et al., 1994). Also in marine fish spermatozoa, this Ca^{2+}/CaM dependent-protein mechanism is essential for sperm motility triggering (Zilli et al., 2017), which may elucidate this experiment results with F1 Senegalese sole sperm motility minor increase.

Altogether, it is worth discerning that sperm motility slight enhancement by melatonin supplementation, in most assessed sperm descriptors in F1 Senegalese sole achieves great relevance, as these parameters are positively correlated with fertilization potential (Rurangwa et al., 2004). This is especially crucial for both F1 Senegalese sole and European eel aquaculture progress. Further exploring melatonin potential effects could be promising for sperm management procedures for industrial application such as broodstock artificial fertilization and sperm cryopreservation. Therefore, melatonin studies in fish spermatozoa should be carried out by assessing sperm motility with different melatonin range concentrations (possibly lower concentrations), or in non- and activating media (e.g. enriched and absent cAMP media would allow to check if this metabolite is indeed involved in sperm motility through melatonin action) and, moreover analysing fish sperm subpopulations exposed to melatonin.

2. Effect of melatonin in post-thaw F1 *Solea senegalensis* sperm quality

Sperm cryopreservation in aquaculture industry has a great potential as it should benefit the management of reproduction of males with high reproductive performance and further permit the long-term storage of their high-quality sperm samples. Various fish sperm cryopreservation protocols have been successfully established for several teleost species thought the development of appropriate quality evaluation methodologies (Cabrita et al., 2014). However, it is well known that sperm cryopreservation procedures can lead to an imbalanced ROS generation and ultimately trigger intrinsic apoptosis pathway. This damage is consequently reflected in a decrease of DNA integrity and spermatozoa viability, which are key factors in sperm quality and, thus progeny success (Bobe and Labbé, 2010). Thus, in order to decrease these impacts of cryopreservation and, consequently enhance post-thawed quality samples, the incorporation of antioxidants in sperm cryopreservation extender media has been assessed (Cabrita et al., 2011a; Martínez-Páramo et al., 2013). Nonetheless, their effects depended on the type of antioxidant, its concentration and can be species-specific (Cabrita et al., 2011b). Therefore, there is a great need to further explore other compounds with similar antioxidant potential for fish sperm cryopreservation success. As previously explained above, melatonin is widely described as a potent antioxidant as it improves cellular physiology and such potential has been demonstrated through remarkable improvements

in other vertebrates post-thawed spermatozoa (Hezavehei et al., 2018), but has never explored in fish sperm cryopreservation.

In this perspective, the second experiment was performed to assess the effect of melatonin supplementation in post-thawed sperm from F1 Senegalese sole, by employing several techniques to evaluate its antioxidant impact in specific oxidative stress markers. For F1 Senegalese sole sperm cryopreservation, the protocol developed by Riesco et al. (2017) was applied and considered as control. In experiment 2, post-thawed sperm motility in control and both melatonin supplementation groups (0.1 and 10 mM) decreased throughout post-activation time. This fast decrease is mostly related to ATP content decline and energy deficiency, worsen by cryopreservation damage (Cabrita et al., 2019; Cosson, 2019). At 15 s post-activation, which is considered the most relevant post-activation time for fertilization, total and progressive motility rates were notably low herein, therefore indicating a general low-quality of sperm. Comparing these results with those obtained by Riesco et al. (2017) in wild breeders, which recorded post-thawed motility rates above 30%, it is possible that low motility values registered are derived from the lower quality of F1 Senegalese sole sperm (Cabrita et al., 2006) and from pooling samples that, as mentioned before, decreases some quality parameters. In similarity, Valcarce and Robles (2016) also exhibited low post-thawed quality in sperm samples obtained from F1 breeders with motility values lower than 30% and disclosed an innate susceptibility in Senegalese sole sperm to suffer oxidative cellular injuries (Valcarce and Robles, 2016).

In this perspective, Valcarce and Robles (2016) also reported that post-thawed sperm from F1 breeders exhibited high DCF positive cells generating hydrogen peroxide and consistently appeared with high ROS levels (Valcarce and Robles, 2016). In the experiment herein, 10 mM melatonin supplement post-thawed sperm also revealed a high percentage of cells producing superoxide anion radical (DHE positive) with ROS detection. Therefore, this suggests that post-thawed sperm with 10 mM melatonin supplemented was more prone to suffer oxidative stress by ROS damage than the remaining experimental groups. Such exposure to ROS can readily trigger the apoptotic cascade characterized by the activation of enzymatic caspases, such as caspases 3 and 7 (Aitken et al., 2012). Indeed, this is verified with the apoptosis detection, whereas 10 mM melatonin concentration also exhibited significantly higher percentages of late apoptotic cells dead by caspase 3/7 enzymatic activity (caspase 3/7 and 7-AAD positive). Thus,

post-thawed sperm submitted to 10 mM melatonin was more susceptible to cellular death by caspase activity and, consequent lethal apoptotic mechanisms. In parallel, the culmination of these events in spermatozoa exposed to 10 mM melatonin were in some way reflected in membrane integrity and DNA fragmentation. Interestingly, the viability percentage in this experimental group was significantly lower than the remaining groups. Similarly, in the comet assay, the 10 mM supplementation also exhibited high percentages of DNA fragmentations. Moreover, in both melatonin concentrations there was a slight decrease of DNA fragmentation percentages comparatively with the control. Altogether, these results suggest that 10 mM melatonin supplement was less efficient in preventing the oxidative stress in post-thawed sperm. Although the percentage of necrotic cells dead by other mechanisms (caspase 3/7 negative, 7-AAD positive) was high with 0.1 mM melatonin supplement, overall, this concentration revealed more promising results. However, such event can be associated with inherent damage induced by cryopreservation due to ice crystal formation that cause membrane disruption (Diogo et al., 2018).

Interestingly, there was one parameter in this experiment that did not follow the same trend. The MDA concentration and, thus lipid peroxidation were reasonably low under 10 mM melatonin supplementation in comparison with 0.1 mM. It has been verified in human spermatozoa that the stimulation of ROS generation ultimately leads to the degradation of membrane PUFAs and, therefore results in lipid peroxidation (Aitken et al., 2012). The supplementation of melatonin with the perspective of reducing such spermatozoa ROS attack, therefore preventing lipid peroxidation, has been confirmed in other species (Appiah et al., 2019b; Mehaisen et al., 2020). However, this impaired result herein among the melatonin concentrations may be due to the ineffectiveness of lipid peroxidation detection by the MDA assay. The applied kit indicates there are interferences in the presence of hydrogen peroxide (H_2O_2) which reduce MDA concentration by 13% (Ge et al., 1998). Indeed, as mentioned previously, there was a high generation of ROS with 10 mM melatonin and these were assessed by superoxide radical (O_2^-) presence in the DHE and SYTOX® green assay. However, there are other radical species, such as hydrogen peroxide, which were not detected with this specific assay but could have been generated by spermatozoa upon cold shock. This might be a possible explanation for the low MDA concentration observed in 10 mM melatonin concentration. Interestingly, the ROS generation in post-thawed Senegalese sole sperm was also assessed by Valcarce and

Robles (2016) using a DCF-DA probe to precisely reveal intracellular hydrogen peroxide formation and was detected by flow cytometry and confocal microscopy. These disclosed the presence of DFH positive cells, triggered mostly by hydrogen peroxide formation, in spermatozoa mitochondria and nucleus (Valcarce and Robles, 2016). Therefore, this techniques and others used for lipid peroxidation assessment should be considered in further studies performed with melatonin supplementation in cryopreservation extender. For instance, BODIPY® fluorescence probe was also used in rooster post-thawed sperm to evaluate lipid peroxidation and showed that melatonin supplementation had a significant effect in minimizing membrane degradation caused by cryopreservation (Mehaisen et al., 2020). Another approach may be through the assessment of antioxidant enzyme activity, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GSR). These antioxidant enzymes were increased in chicken post-thawed sperm with the addition of melatonin in the extender (Appiah et al., 2019a). Altogether, this experiment allowed to gain insights regarding melatonin as a possible supplement in F1 Senegalese sole post-thawed sperm and it permits to consider that melatonin might have more promising results in lower concentrations. Although it is difficult to overcome the low sperm volume collected and the application of specific techniques, a more deep analysis of the effects of melatonin, especially at mitochondrial potential level and other ROS generation pathways, should be performed in further studies.

Conclusions

- The supplementation of melatonin at different concentration and during various times of exposure did not produce any significant effect on European eel (*A. anguilla*), mostly due to this species tolerance to methanol.

- The supplementation of melatonin at different concentration in F1 Senegalese sole (*S. senegalensis*) sperm exhibited time exposure effect. In addition, both concentrations 0.1 and 10 mM showed a slight increase in sperm motility in *S. senegalensis*.

- The sperm cryopreservation protocol established by Riesco et al. (2017) applied with melatonin supplementation in the extender media did not improve post-thawed F1 Senegalese sole sperm.

- The supplementation of the cryopreservation extender with melatonin at 10 mM revealed more spermatozoa damage and, therefore, less protection than 0.1 mM in F1 Senegalese sole. Thus, lower concentrations and new sperm quality evaluation tools should be considered in further studies.

References

Acuña, D., Germaine, C., Carmen, E., Díaz, M.E., Elena, C., Cabello, L., López, L.C., Rosales, S., Dun, C., Tan, X., Reiter, R.J., 2014. Extrapineal melatonin: sources, regulation, and potential functions. Cell. Mol. Life Sci. 71, 2997–3025.

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function - in sickness and in health. J. Androl. 33, 1096–1106.

Aitken, R.J., Koppers, A.J., 2011. Apoptosis and DNA damage in human spermatozoa. Asian J. Androl. 13, 36–42.

Alavi, S.M.H., Cosson, J., Bondarenko, O., Linhart, O., 2019. Sperm motility in fishes: (III) diversity of regulatory signals from membrane to the axoneme. Theriogenology 136, 143–165.

Alavi, S.M.H., Linhart, O., Coward, K., Rodina, M., 2007. Fish spermatology: Implications for aquaculture management, in: Alavi, S.M.H., Cosson, J., Raffie, G. (Eds.), Fish Spermatology. Alpha Science Intl Ltd, Oxford, UK, pp. 397–460.

Andersson, T.P.M., Svensson, S.P.S., Karlsson, A.M., 2003. Regulation of melanosome movement by MAP kinase. Pigment Cell Res. 16, 215–221.

Appiah, M.O., He, B., Lu, W., Wang, J., 2019a. Antioxidative effect of melatonin on cryopreserved chicken semen. Cryobiology 89, 90–95.

Appiah, M.O., He, B., Lu, W., Wang, J., 2019b. Antioxidative effect of melatonin on cryopreserved chicken semen. Cryobiology 89, 90–95.

Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S., 2005. Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. Neuroendocrinology 82, 221–232.

Ashrafi, I., Kohram, H., Ardabili, F.F., 2013. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim. Reprod. Sci. 139, 25–30.

Asturiano, J.F., Pérez, L., Barrera, R., Jover, M., 2004. Producción de anguilas: pasado, presente y futuro. Aquat. Rev. electrónica Acuic. 51–78.

Balao Da Silva, C.M., MacÍas-García, B., Miró-Morán, A., González-Fernández, L., Morillo-Rodriguez, A., Ortega-Ferrusola, C., Gallardo-Bolaños, J.M., Stilwell, G., Tapia, J.A., Peña, F.J., 2011. Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. J. Pineal Res. 51, 172–179.

Beirão, J., 2011. Sperm quality in marine teleosts: applications to broodstock management and sperm storage in two farmed species *Solea senegalensis* and *Sparus aurata*. Dr. Thesis, Dpto. Biol. Mol. Fac. C.C. Biológicas y Ambient.

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., Herráez, M.P., 2011a. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 62, 22–31.

Beirão, J., Cabrita, E., Soares, F., Herráez, M.P., Dinis, M.T., 2008. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: Comet analysis and Annexin V-Fluorescein staining. J. Appl. Ichthyol. 24, 508–513.

Beirão, J., Pérez-Cerezales, S., Martínez-Páramo, S., Herráez, M.P., 2010. Detection of early damage of sperm cell membrane in Gilthead seabream (*Sparus aurata*) with the nuclear stain YO-PRO 1. J. Appl. Ichthyol. 26, 794–796.

Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2011b. Changes in *Solea senegalensis* sperm quality throughout the year. Anim. Reprod. Sci. 126, 122–129.

Benítez-King, G., Ortiz-López, L., Jiménez-Rubio, G., 2005. Melatonin precludes cytoskeletal collapse caused by hydrogen peroxide: participation of protein kinase C. Therapy 2.

Bobe, J., Labbé, C., 2010. General and comparative endocrinology egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535–548.

Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 63–98.

Buffone, M., Wertheimer, E., Visconti, P., Krapf, D., 2015. Central role of soluble adenylyl cyclase and cAMP in sperm physiology. Biochim. Biophys. Acta 1842, 2610–2620.

Cabrita, E., Alvarez, R., Anel, L., Rana, K.J., Herraez, M.P., 1998. Sublethal Damage during Cryopreservation of Rainbow Trout Sperm. Cryobiology 37, 245–253.

Cabrita, E., Herráez, P., Robles, V., 2008a. Sperm quality assessment, in: Cabrita, E., Robles, V., Herráez, P. (Eds.), Methods in Reproductive Aquaculture - Marine and Freshwater Species. CRC Press/Taylor & Francis, Boca Raton, pp. 93–148.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., Dinis, M.T., 2011a. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. Anim. Reprod. Sci. 125, 189–195.

Cabrita, E., Martínez-Páramo, S., Diogo, P., Sarasquete, C., Dinis, M.T., 2011b. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. Anim. Reprod. Sci. 125, 189–195.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432, 389–401.

Cabrita, E., Riesco, M.F., Manãnós, E., 2019. Sperm Physiology and Artificial Fertilization, in: Muñoz-Cueto, J.A., Manãnós, E., Sánchez-Vázquez, F.J. (Eds.), The Biology of Sole. CRC Press/Taylor & Francis, pp. 143–168.

Cabrita, E., Robles, V., Cuñado, S., Wallace, J.C., Sarasquete, C., Herráez, M.P., 2005. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5ml macrotubes. Cryobiology 50, 273–284.

Cabrita, E., Robles, V., Herraez, P., 2008b. Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC Press/Taylor & Francis.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010a. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichthyol. 26, 623–635.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010b. Cryopreservation of fish sperm: Applications and perspectives. J. Appl. Ichthyol. 26, 623–635.

Cabrita, E., Soares, F., Dinis, M.T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261, 967–975.

Caldeira, C., Hernández-ibáñez, S., Valverde, A., Martin, Patrick, Herranz-jusdado, J.G., Gallego, V., Asturiano, J.F., Dzyuba, B., Martin, P, 2019. Standardization of sperm motility analysis by using CASA-Mot for Atlantic salmon (*Salmo salar*), European eel (*Anguilla anguilla*) and Siberian sturgeon (*Acipenser baerii*) 502, 223–231.

Carvalho, A.F.S., Machado, M.R.F., Andrade, E.S., Murgas, L.D.S., Zangeronimo, M.G., Barros, T.C., Paula, F.G., 2014. Effect of caffeine added to the activating solution on sperm motility of fresh and thawed semen of pacu, *Piaractus mesopotamicus*, and curimba, *Prochilodus lineatus*. J. World Aquac. Soc. 45, 75–

Cebrián-Pérez, J., Casao, A., Gonzalez-Arto, M., Hamilton, T., Pérez-Pé, R., Muiño-Blanco, T., 2014. Melatonin in sperm biology: breaking paradigms. Reprod. Domest. Anim. 4, 11–21.

Cosson, J., 2019. Fish Sperm Physiology: structure, factors regulating motility, and motility evaluation, in: Bozkurt, Y. (Ed.), Biological Research in Aquatic Sciences. Intech Open, pp. 1–27.

Deng, S., Sun, T., Yu, K., Wang, P., Zhang, B., Zhang, Y., Lian, Z., Liu, Y., 2017. Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen, Free Radical Biology and Medicine.

Dinis, M.T., 1992. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. Aquac. Res. 23, 515–520.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27–38.

Diogo, P., Martins, G., Quinzico, I., Nogueira, R., Gavaia, P.J., Cabrita, E., 2018. Electric ultrafreezer (− 150 °C) as an alternative for zebrafish sperm cryopreservation and storage. Fish Physiol. Biochem. 44, 1443–1455.

Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., Prokopchuk, G., Cosson, J., 2017. Energetics of fish spermatozoa: The proven and the possible. Aquaculture 472, 60–72.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469–482.

Fang, Y., Zhao, C., Xiang, H., Zhao, X., Zhong, R., 2020. Melatonin inhibits formation of mitochondrial permeability transition pores and improves oxidative phosphorylation of frozen-thawed ram sperm. Front. Endocrinol. (Lausanne). 10, 1–13.

FAO, 2018. The State of World Fisheries and Aquaculture: Meeting the sustainable development goals. Rome.

Fernández-Alegre, E., Álvarez-Fernandez, Carlos, J., Casao, A., Mart, F., 2020. Melatonin non-linearly modulates Bull spermatozoa motility and physiology in capacitating and non-capacitating conditions. Int. J. Mol. Sci. 21, 1–20.

Ferramosca, A., Pinto Provenzano, S., Montagna, D.D., Coppola, L., Zara, V., 2013. Oxidative stress negatively affects human sperm mitochondrial respiration. Urology 82, 78–83.

Ferreira, H., Ramalho, A., Dias, J., Yúfera, M., Arias, A., Falcão, M., Serpa, D., Aires, T., Pousão-Ferreira, P., Cunha, M.E., Valente, L.M.P., Dinis, M.T., Conceição, L., 2010. Sustainable semi-intensive polyculture of seabream and sole in earthen ponds. Aquac. Eur. 35, 17–21.

Figueroa, E., Valdebenito, I., Farias, J.G., 2016. Technologies used in the study of sperm function in cryopreserved fish spermatozoa. Aquac. Res. 47, 1691–1705.

Francis, F., Ramanathan, N., Padmavathy, P., 2004. Role of the pineal organ and melatonin in fish. World Aquac. 21–24.

Freyhof, J., Kottelat, M., 2010. *Anguilla anguilla*. The IUCN Red List of Threatened Species [WWW Document].

Fujinoki, M., 2008. Melatonin-enhanced hyperactivation of hamster sperm. Reproduction 136, 533–541.

Gallego, V., Asturiano, J.F., 2018. Sperm motility in fish: technical applications and perspectives through CASA-Mot systems. Reprod. Fertil. Dev. 30, 820–832.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2014. Sperm motility parameters and spermatozoa morphometric characterization in marine species: A study of swimmer and sessile species. Theriogenology 82, 668–676.

Gao, Y., Yang, C., Huang, C., Zhang, G., Ji, H., Dong, W., 2019. The effects of melatonin supplement on paddle fish (*Polyodon spathula*) sperm quality and ATP content in sperm during in vitro storage. Aquaculture 503, 475–482.

Ge, D., Erdelmeier, I., Re, K., Moze-henry, N., Yadan, J., Chaudie, J., 1998. Reactions of 1-Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Analytical Applications to a colorimetric assay of lipid peroxidation. Chem. Res. Toxicol. 11, 1176–1183.

Gimeno-Martos, S., Casao, A., Yeste, M., Cebrián-Pérez, J.A., Muiño-Blanco, T., Pérez-Pé, R., 2019. Melatonin reduces cAMP-stimulated capacitation of ram spermatozoa. Reprod. Fertil. Dev. 31, 420–431.

González-Arto, M., Aguilar, D., Gaspar-Torrubia, E., Gallego, M., Carvajal-Serna, M., Herrera-Marcos, L., Serrano-Blesa, E., Hamilton, T., Pérez-Pé, R., Muiño-Blanco, T., Cebrián-Pérez, J., Casao, A., 2017. Melatonin MT1 and MT2 receptors in the ram reproductive tract. Int. J. Mol. Sci. 18, 662.

Gonzalez-Arto, M., Vicente-Carrillo, A., Martinez-Pastor, F., Fernández-Alegre, E., Roca, J., Rigau, T., Rodriguez-Gil, J., Pérez-Pé, R., Muino-Blanco, T., Cebrián-pérez, J.A., Casao, A., 2016. Melatonin receptors MT1 and MT2 are expressed in spermatozoa from several seasonal and non-seasonal breeder species, Theriogenology.

Guerra, S.M., Valcarce, D.G., Cabrita, E., Robles, V., 2013. Analysis of transcripts in gilthead Seabream sperm and zebrafish testicular cells: mRNA profile as a predictor of gamete quality. Aquaculture 406–407, 28–33.

Gurer-Orhab, H., Suzen, S., 2015. Melatonin, its metabolites and its synthetic analogs as multi-faceted compounds: antioxidant, prooxidant and inhibitor of bioactivation reactions. Curr. Med. Chem. 22, 490–499.

Hagedorn, M., McCarthy, M., Carter, V.L., Meyers, S.A., 2012. Oxidative stress in zebrafish (*Danio rerio*) sperm. PLoS One 7, 2–12.

Herráez, M.P., Ausió, J., Devaux, A., González-rojo, S., Fernández-díez, C., Bony, S., Saperas, N., Robles, V., 2017. Patternal contribution to development: Sperm genetic damage and repair in fish. Aquaculture 472, 45–59.

Herranz-Jusdado, J.G., Gallego, V., Morini, M., Rozenfeld, C., Pérez, L., Kása, E., Kollár, T., Depincé, A., Labbe, C., Horváth, Á., Asturiano, J.F., 2019a. Comparison of European eel sperm cryopreservation protocols with standardization as a target. Aquaculture 498, 539–544.

Herranz-Jusdado, J.G., Gallego, V., Rozenfeld, C., Morini, M., Pérez, L., Asturiano, J.F., 2019b. European eel sperm storage: Optimization of short-term protocols and cryopreservation of large volumes. Aquaculture 506, 42–50.

Herranz-Jusdado, J.G., Kása, E., Kollár, T., Gallego, V., Peñaranda, D.S., Rozenfeld, C., Pérez, L., Horváth, Á., Asturiano, J.F., 2018. Handling and Treatment of Male European Eels (*Anguilla anguilla*) for Hormonal Maturation and Sperm Cryopreservation. J. Vis. Exp. 2–7.

Herranz-Jusdado, J.G., Rozenfeld, C., Morini, M., Pérez, L., Asturiano, J.F., Gallego, V., 2019c. Recombinant vs purified mammal gonadotropins as maturation hormonal treatments of European eel males. Aquaculture 501, 527–536.

Hezavehei, M., Sharafi, M., Kouchesfahani, H.M., Henkel, R., Agarwal, A., Esmaeili, V., Shahverdi, A., 2018. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod. Biomed. Online 37, 327–339.

Horváth, Á., Urba, B., 2000. The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. Aquac. Res. 31, 317–324.

Horváth, Á., Wayman, W.R., Dean, J.C., Urbányi, B., Tiersch, T.R., Mims, S.D., Johnson, D., Jenkins, J.A., 2008. Viability and fertilizing capacity of cryopreserved sperm from three North American acipenseriform species: a retrospective study. J. Appl. Ichthyol. 24, 443–449.

Huerto-Delgadillo, L., Antón-Tay, F., Benítez-King, G., 1994. Effects of melatonin on microtubule assembly depend on hormone concentration: role of melatonin as a calmodulin antagonist. J. Pineal Res. 17, 55–62.

ICES, 2012. Report of the 2012 Session of the Joint EIFAAC/ICES Working Group on Eels.

Jang, H.., Kim, Y.., Kim, B.., Park, I.., Cheong, H.., Kim, J.., Park, C.., Kong, H.., Lee, H.., Yang, B.., 2010. Ameliorative effects of melatonin against hydrogen peroxide-induced

oxidative stress on boar sperm characteristics and subsequent *in vitro* embryo development. Reprod. Domest. Anim. 950, 943–950.

Jia, G., Meng, Q., 2016. Effect of melatonin supplementation on cryopreserved sperm quality in mouse. CryoLetters 37, 115–122.

Kása, E., Bernáth, G., Kollár, T., Zarski, D., Lujic, J., Bokor, Z., Hegyi, Á., Urbányi, B., Vílchez, M.C., Morini, M., Peñaranda, D.S., Pérez, L., Asturiano, J.F., Horváth, Á., 2017. General and comparative endocrinology development of sperm vitrification protocols for freshwater fish (Eurasian perch, *Perca fluviatilis*) and marine fish (European eel, *Anguilla anguilla*) 245, 102–107.

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130, 425–433.

Len, J.S., Koh, W.S.D., Tan, S.-X., 2019. The roles of reactive oxygen species and antioxidants in cryopreservation. Biosci. Rep. 39, BSR20191601.

Li, C., Hao, H., Zhao, Y., Zhang, P., Wang, H., 2019. Melatonin improves the fertilization capacity of sex-sorted bull sperm by inhibiting apoptosis and increasing fertilization capacitation via MT1. Int. J. Mol. Sci. 20, 1–19.

Li, C., Zhou, X., 2015. Melatonin and male reproduction. Clin. Chim. Acta 446, 175–180.

Liu, L., Labani, N., Cecon, E., Jockers, R., Vaudry, H., Thompson, M.D., Boutin, J.A., Jockers, R., 2019. Melatonin target proteins: too many or not enough ? Front. Endocrinol. (Lausanne). 10, 1–13.

Lombardo, F., Gioacchini, G., Fabbrocini, A., Candelma, M., Adamo, R.D., Giorgini, E., Carnevali, O., 2014. Melatonin-mediated effects on killi fish reproductive axis. Comp. Biochem. Physiol. Part A 172, 31–38.

Lubzens, E., Labbé, C., Ciereszko, A., 2017. Introduction to the special issue on recent advances in fish gamete and embryo Research. Aquaculture 472, 1–3.

Luchetti, F., Canonico, B., Betti, M., Arcangeletti, M., Pilolli, F., Piroddi, M., Canesi, L., Papa, S., Galli, F., 2010. Melatonin signaling and cell protection function. FASEB J. 24, 3603–3624. https://doi.org/10.1096/fj.10-154450

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E., 2012a. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Anim. Reprod. Sci. 131, 211–218.

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E., 2012b. Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. Theriogenology 77, 1129–1136.

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Soares, F., Sarasquete, C., Cabrita, E., 2013. Effect of two sulfur-containing amino acids, taurine and hypotaurine in European sea bass (*Dicentrarchus labrax*) sperm cryopreservation. Cryobiology 66, 333–338.

Martínez-Páramo, S., Pérez-Cerezales, S., Gómez-Romano, F., Blanco, G., Sánchez, J.A., Herráez, M.P., 2009. Cryobanking as tool for conservation of biodiversity : Effect of brown trout sperm cryopreservation on the male genetic potential. Theriogenology 71, 594–604.

Martínez-Pastor, F., Mata-Campuzano, M., Álvarez-Rodríguez, M., Álvarez, M., Anel, L., de Paz, P., 2010. Probes and techniques for sperm evaluation by flow cytometry. Reprod. Domest. Anim. 45, 67–78.

Mehaisen, G.M.K., Partyka, A., Ligocka, Z., Niżański, W., 2020. Cryoprotective effect of melatonin supplementation on post-thawed rooster sperm quality. Anim. Reprod. Sci. 212, 1–7.

Morais, S., Aragão, C., Cabrita, E., Conceição, L.E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L.M.P., Yúfera, M., Dinis, M.T., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Rev. Aquac. 8, 227–263.

Morini, M., Fatsini, E., Ferrão, L., Gallego, V., Frias, P., Oliveira, C., Cabrita, E., 2019. Day and night gene expression of sperm quality markers in Senegalese sole - 7th International Workshop on Biology of Fish Gametes, Rennes, 2019.

Muchlisin, Z.A., 2005. Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. Biodiversitas J. Biol. Divers. 6, 12–15.

Muñoz-Cueto, J., Manãnós, E., Sánchez-Vázquez, F.J., 2019. Reproductive physiology and broodstock management of Sole, in: Muñoz-Cueto, J.A., Manãnós, Evaristo, Sánchez-Vázquez, Francisco Javier (Eds.), The Biology of Sole. CRC Press/Taylor & Francis, pp. 90–141.

Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2017. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472, 21–44.

Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516–534.

Najafi, A., Adutwum, E., Yari, A., Salehi, E., Mikaeili, S., Dashtestani, F., Abolhassani, F., Rashki, L., Shiasi, S., Asadi, E., 2018. Melatonin affects membrane integrity, intracellular reactive oxygen species, caspase3 activity and AKT phosphorylation in frozen thawed human sperm. Cell Tissue Res. 372, 149–159.

Oliveira, C., Dinis, M.T., Soares, F., Cabrita, E., Pousão-Ferreira, P., Sánchez-Vázquez, F.J., 2009. Lunar and daily spawning rhythms of Senegal sole *Solea senegalensis*. J. Fish Biol. 75, 61–74.

Oliveira, C., Duncan, N.J., Pousão-Ferreira, P., Mañanós, E., Sánchez-Vázquez, F.J., 2010. Influence of the lunar cycle on plasma melatonin, vitellogenin and sex steroids rhythms in Senegal sole, *Solea senegalensis*. Aquaculture 306, 343–347.

Oliveira, C., Mañanós, E., Ramos, J., Sánchez-Vázquez, F.J., 2011. Impact of photoperiod manipulation on day/night changes in melatonin, sex steroids and vitellogenin plasma levels and spawning rhythms in Senegal sole, *Solea senegalensis*. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 159, 291–295.

Oliveira, C., Sánchez-Vázquez, F.J., 2010. Reproduction Rhytms in Fish, in: Kulczykowska, E., Popek, W., Kapoor, B.. (Eds.), Biological Clock in Fish. CRC Press/Taylor & Francis, pp. 185–216.

Ortiz, Á., Espino, J., Bejarano, I., Lozano, G., Monllor, F., Pariente, J., 2011. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J. Pineal Res. 50, 132–139.

Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010. European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 415, 407–415.

Pérez-Cerezales, S., Gutiérrez-Adán, A., Martínez-Páramo, S., Beirão, J., Herráez, M.P., 2011. Altered gene transcription and telomere length in trout embryo and larvae obtained with DNA cryodamaged sperm. Theriogenology 76, 1234–1245.

Reiter, R.J., Rosales-Corral, S., Tan, D.X., Jou, M.J., Galano, A., Xu, B., 2017. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. Cell. Mol. Life Sci. 74, 3863–3881.

Riesco, M.F., Oliveira, C., Soares, F., Gavaia, P.J., Cabrita, E., 2017. *Solea senegalensis* sperm cryopreservation: New insights on sperm quality. PLoS One 12, 1–19.

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Martín, I., Calderón-García, A.Á., Gonzalez-Nunez, V., Robles, V., 2019. Male reproductive dysfunction in *Solea senegalensis*: New insights into an unsolved question. Reprod. Fertil. Dev.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1–28.

Said, T.M., Gaglani, A., Agarwal, A., 2010. Implication of apoptosis in sperm cryoinjury. Reprod. Biomed. Online 21, 456–462.

Słowińska, M., Nynca, J., Cejko, B.I., Dietrich, M.A., Horváth, Á., Urbányi, B., Kotrik, L., Ciereszko, A., 2013. Total antioxidant capacity of fish seminal plasma. Aquaculture 400–401, 101–104.

Stone, R., 2003. Freshwater eels are slip sliding away. Science (80). 221–222.

Suquet, M., Cosson, J., Donval, A., Labbé, C., Boulais, M., Haffray, P., Bernard, I., Fauvel, C., 2012. Marathon vs sprint racers: An adaptation of sperm characteristics to the reproductive strategy of Pacific oyster, turbot and seabass. J. Appl. Ichthyol. 28, 956–960.

Szabó, G., Müller, T., Bercsényi, M., Urbányi, B., Kucska, B., Horváth, Á., 2005. Cryopreservation of European eel (*Anguilla anguilla*) sperm using different extenders and cryoprotectants. Acta Biol. Hung. 56, 173–175.

Tesch, F.W., 2003. The eel, 3th ed, The Fisheries Society of the British Isles. Blackweel Science, Oxford.

Tiersch, T.R., Yang, H., Jenkins, J.A., Dong, Q., 2007. Sperm cryopreservation in fish and shellfish. Soc. Reprod. Fertil. 65, 493–508.

Tsukamoto, K., Chow, S., Otake, T., Kurogi, H., Mochioka, N., Miller, M.J., Aoyama, J., Kimura, S., Watanabe, S., Yoshinaga, T., Shinoda, A., Kuroki, M., Oya, M., Watanabe, T., Hata, K., Ijiri, S., Kazeto, Y., Nomura, K., Tanaka, H., 2011. Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat. Commun. 2, 9.

Valcarce, D.G., Herráez, M.P., Chereguini, O., Rodríguez, C., Robles, V., 2016. Selection of nonapoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*). Theriogenology 1–8.

Valcarce, D.G., Robles, V., 2016. Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa. Reproduction 152, 439–446.

Xin, M., Niksirat, H., Shaliutina-Kolešová, A., Siddique, M.A.M., Sterba, J., Boryshpolets, S., Linhart, O., 2019. Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation. Rev. Aquac.

Zhao, D., Yu, Y., Shen, Y., Liu, Q., Zhao, Z., Sharma, R., Reiter, R.J., 2019. Melatonin synthesis and function: Evolutionary history in animals and plants. Front. Endocrinol. (Lausanne). 10, 1–16.

Zilli, L., Schiavone, R., Vilella, S., 2017. Role of protein phosphorylation/ dephosphorylation in fish sperm motility activation: state of art and perspectives. Aquaculture 472, 73–80.